Janthinobacterium agaricidamnorum* sp. nov., a soft rot pathogen of *Agaricus bisporus

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A novel bacterium has been found that causes a soft rot disease of *Agaricus bisporus*, the cultivated mushroom. It has been characterized using nutritional, physiological, chemical and molecular techniques. Based on these data, it was shown to have many characteristics in common with members of the genus *Janthinobacterium*. Despite similarities to the only described species within this genus, *Janthinobacterium lividum*, there were a number of differences between the mushroom pathogen isolated and this species. Despite the high degree of genotypic similarity between members of the genus *Janthinobacterium* and *Herbaspirillum*, as evidenced by DNA–RNA hybridization, and the high degree of 16S rDNA sequence similarity between members of the genera *Janthinobacterium*, *Herbaspirillum*, *Oxalobacter* and *Duganella*, as well as the generically misnamed *Pseudomonas lemoignei*, it was possible to show that members of the genus *Janthinobacterium* could be easily distinguished from these taxa. The data also indicated that the mushroom pathogenic strains represent a novel species within the genus *Janthinobacterium* for which the name *Janthinobacterium agaricidamnorum* sp. nov. is proposed. The type strain of this species has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, as DSM 9628^T and at the National Collection of Plant-pathogenic bacteria, UK, as NCPPB 3945^T. To aid practical control of the disease, the effect of the relative humidity on symptom expression on *Agaricus bisporus* was determined.

Keywords: *Janthinobacterium agaricidamnorum*, *Agaricus bisporus* pathogen, taxonomy, 16S rDNA sequence, chemotaxonomy

INTRODUCTION

A soft rot disease has caused localized devastation of a crop of *Agaricus bisporus* (Sinden Hauser 3.8), a brown strain of the cultivated mushroom, on a farm in southern England. Some mushrooms dissolved completely *in situ* within 2 d of symptoms becoming apparent. Other, less dramatic, symptoms included pitting and sticky blotches on the mushroom cap surface. Disease outbreaks showing similar symptoms, have been reported from cave farms in France and with increasing frequency from British mushroom farms.

Abbreviation: RH, relative humidity.

The EMBL accession number for the 16S rDNA sequence of *Janthinobacterium agaricidamnorum* sp. nov. is Y08845.

The symptoms are similar to those of rapid rot of *Agaricus bitorquis*, grown at a higher temperature (28 °C), which is caused by *Burkholderia gladioli* pv. *agaricicola* (Lincoln *et al.*, 1991). The causal organism of the new *A. bisporus* disease has been isolated and characterized using a combination of phenotypic (standard biochemical and nutritional bacteriological tests in harness with chemical profiling) and genotypic (G+C base composition of total genomic DNA, DNA–DNA hybridization and 16S rDNA sequence analysis) techniques. A high degree of similarity was established between members of the genera *Janthinobacterium*, *Oxalobacter*, *Duganella* and *Herbaspirillum*, together with the generically misnamed species *Pseudomonas lemoignei*, as evidenced by DNA–RNA hybridization studies and 16S rDNA sequence analysis. On the basis of this information, it was necessary to consider, together with phenotypic

data, whether the genus *Janthinobacterium* should be expanded to include those members of the genera listed above. The use of whole-cell fatty acid analysis was found particularly useful for distinguishing members of the genus *Janthinobacterium* from other, closely related genera. The mushroom pathogenic strains could be shown to be members of the genus *Janthinobacterium* based on 16S rDNA sequence and fatty acid analysis. However, DNA-DNA hybridization and comparative biochemical studies of these isolates and members of the species *Janthinobacterium lividum* indicated that the mushroom pathogens constitute a novel species within the genus *Janthinobacterium*. In the light of these findings, it was considered appropriate to emend the description of the genus *Janthinobacterium*.

On the basis of the taxonomic study, further studies on the pathogenicity of the members of the genus *Janthinobacterium* were undertaken. A small mushroom crop was grown to investigate environmental effects on symptom expression and to check anecdotal evidence that brown *Agaricus* strains were more susceptible to the pathogen than white strains. The bacterial diseases normally associated with mushrooms are brown blotch, caused by *Pseudomonas tolaasii* (Paine, 1919); ginger blotch, caused by '*Pseudomonas gingeri*' (Wong *et al.*, 1982), and drippy gill, caused by *Pseudomonas agarici* (Young, 1970). This is the first bacterial disease of *A. bisporus* to be caused by a species of the genus *Janthinobacterium*. Other species causing bacterial diseases of mushrooms which are not members of the genus *Pseudomonas* are *B. gladioli* pv. *agaricola* (Lincoln *et al.*, 1991) and *Ewingella americana* (internal stipe necrosis) (Inglis *et al.*, 1996).

METHODS

Bacterial cultures. Species and strains of bacteria compared in this study are listed in Table 1.

Samples of diseased mushroom tissue were transferred aseptically to sterile quarter-strength Ringer's solution and a ten-fold dilution series was inoculated onto *Pseudomonas* agar F (PAF; Merck) and nutrient agar (Oxoid) and incubated at 25 °C for 24 h. Single colonies were isolated from the dilution plates using a sterile wire and streaked onto plates of the appropriate agar and cultured at 25 °C. Each isolate was 'white line tested' for identity as *P. tolaasii* (Wong & Preece, 1979), tested on mushroom tissue blocks as an initial pathogenicity test (Gandy, 1968) and plated onto *Pseudomonas* isolation agar (PIA; Difco). Two isolates produced severe symptoms; these were designated W1r3^T (= NCPPB 3945^T = DSM 9628^T) and C1b4 (= NCPPB 3965) and were from different mushroom houses on the same farm. W1r3^T was from a rotting mushroom and C1b4 was from a blotched mushroom. Both of these isolates were applied to growing mushrooms at Horticulture Research International to check pathogenicity. Soft rot bacterium H5 (= NCPPB 3964) was isolated from *A. bisporus* on a commercial mushroom farm in France and was a gift from J. M. Olivier, INRA Bordeaux. Difficulty was experienced in maintaining cultures of the mushroom pathogens on agar as the cultures died if left for more than 10 d without subculturing. The

isolates were stored freeze-dried and in liquid nitrogen. Working cultures were subcultured weekly.

Characterization and identification of the causal organism.

Test organisms were subjected to a number of biochemical tests (Gerhardt *et al.*, 1981; Lelliott & Stead, 1987). All strains were subjected to biochemical testing in the API 20NE, API 50CH and the BIOLOG GN microtitre plate systems. All strains were pre-cultivated on nutrient agar for 24 h at 28 °C and resuspended in the appropriate test media according to the manufacturer's instructions.

Chemical characterization. All strains of the genera *Janthinobacterium* and *Herbaspirillum* used in the present chemotaxonomic study were grown in nutrient broth at 28 °C for 24 h, shaken at 100 r.p.m. on an orbital shaker. Cells were harvested by centrifugation at 10000 r.p.m. for 20 min. Cell pellets were freeze-dried and the dried material was stored at -20 °C until used for the analyses.

Whole-cell fatty acid analysis (Moss *et al.*, 1980) of the new pathogenic isolates was performed at MAFF Central Science Laboratory (CSL), Harpenden, UK, as in Lincoln *et al.* (1991), and at the DSMZ Braunschweig, Germany (all strains), where methods were employed which allowed selective hydrolysis of ester- and amide-linked fatty acids (B. J. Tindall, unpublished results).

Extraction and analysis of respiratory lipoquinones and polar lipids. Respiratory lipoquinones and polar lipids were extracted from 100 mg freeze-dried material using the two-stage method described by Tindall (1990a, b).

Respiratory lipoquinones were separated into their different classes (menaquinones and ubiquinones) by TLC; UV-absorbing bands, corresponding to menaquinones or ubiquinones, were removed from the plate and analysed further by HPLC as described by Tindall (1990a, b).

Polar lipids were separated by two-dimensional silica gel TLC (Macherey-Nagel) as described by Tindall (1990a, b). Total lipid material was detected using dodecamolybdophosphoric acid and specific functional groups were detected using Zinzadze reagent (phosphate), ninhydrin (free amino groups), periodate-Schiff (α -glycols), Dragendorff (quaternary nitrogen), anisaldehyde-sulphuric acid (glycolipids) and α -naphthol-sulphuric acid (glycolipids).

Resistance to antibiotics. Antibiotic resistance of the mushroom pathogenic strains and the *J. lividum* strains was tested on plates of Iso-sensitest agar (Oxoid) pre-inoculated with the test organism and dried. Antimicrobial susceptibility test discs (Oxoid) were placed on the agar surface. These included penicillin G (10 µg disc), vancomycin (30 µg disc), erythromycin (15 µg disc), streptomycin (10 µg disc), tetracycline (30 µg disc) and nalidixic acid (30 µg disc). The plates were then incubated at 25 °C for 48 h. If the area surrounding a disc was free from bacterial growth, it was recorded that the organism was sensitive to that antibiotic.

DNA extraction and PCR amplification of the 16S rDNA.

Genomic DNA extraction, PCR-mediated amplification of the 16S rDNA and purification of PCR products was carried out as described previously (Rainey & Stackebrandt, 1993; Rainey *et al.*, 1992a). Purified PCR products were sequenced using the *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) as directed in the manufacturer's protocol. Sequence reactions were electrophoresed using the Applied Biosystems 373A DNA Sequencer. The 16S rDNA sequences were aligned manually. The global phylogenetic position of strain W1r3^T was investigated using the ARB database (Ludwig & Strunk, 1996). On the basis of this

Table 1. Species and strains of bacteria compared in this study

Name	Culture collection no.*	No. used in this study	Source
<i>Janthinobacterium agaricidamnosum</i>	NCPPB 3945 ^T (= DSM 9628 ^T)	W1r3 ^T	Rotting <i>A. bisporus</i> , UK
<i>Janthinobacterium agaricidamnosum</i>	NCPPB 3965	C1b4	Blotches <i>A. bisporus</i> , UK
<i>Janthinobacterium agaricidamnosum</i>	NCPPB 3964	H5†	Rotting <i>A. bisporus</i> , France
<i>Janthinobacterium lividum</i>	NCIMB 9230		Soil, UK
<i>Janthinobacterium lividum</i>	NCIMB 9414		Spring water, UK
<i>Janthinobacterium lividum</i>	DSM 1522 ^T		Soil, USA
<i>Herbaspirillum seropedicae</i>	DSM 6445 ^T		<i>Oryza sativa</i> roots, Brazil
<i>Herbaspirillum rubrisubalbicans</i>	DSM 9940 ^T		<i>Saccharum officinarum</i> , USA
<i>Herbaspirillum</i> sp.	DSM 11581 (= LMG 2285)		<i>Saccharum officinarum</i> , Australia
<i>Herbaspirillum</i> sp.	DSM 11582 (= LMG 5321)		Leg wound, Sweden
<i>Herbaspirillum</i> sp.	DSM 11580 (= LMG 5523)		Contaminant, Sweden

* NCPPB, National Collection of Plant-pathogenic Bacteria, Central Science Laboratory, York, UK; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, UK; LMG, Collection of Bacteria of the Laboratorium voor Microbiologie, K.-L. Ledengankstraat, Gent, Belgium. T, Type strain.

† Gift from J. M. Olivier, INRA, Bordeaux, France.

work, the 16S rDNA sequences were compared with the existing 16S rDNA database for members of the phyletic group defined as the β -subclass of the *Proteobacteria*. A greater degree of resolution among closely related taxa was performed using the ae2 editor (Maidak *et al.*, 1997). Similarity values were calculated based on the aligned 16S rDNA sequences from the following organisms: *J. lividum* DSM 1522^T (Y08846); *Telluria mixta* (X65589); *Telluria chitinolytica* (X65590); *Duganella zoogloeoides* IAM 12670^T (D14256); *Oxalobacter formigenes* strain OXB^T (U49757); *P. lemoignei* LMG 2207^T (X92555); *Herbaspirillum seropedicae* DSM 6445^T (Y10146). Strain designations for *T. mixta* and *T. chitinolytica* were not given in the EMBL database, although in the work of Bowman *et al.* (1993), in which these sequences were determined, only *T. mixta* ACM 1762^T (= ATCC 49108^T) and *T. chitinolytica* 20M^T (= ACM 3522^T = CNCM I-804^T) were used.

DNA base composition. The percentage G + C content in the DNA was estimated using a DNA mid-thermal melting point method (Marmur & Doty, 1962). DNA was extracted from the bacteria using the method of Neumann *et al.* (1992).

DNA-DNA hybridization. DNA was isolated by passing the cell suspensions through a French press and purified on a hydroxyapatite column according to the method of Cashion *et al.* (1977). Prior to hybridization, the G + C base content of the two *Janthinobacterium* strains was assessed by hydrolysis of the purified DNA with P1 nuclease and dephosphorylation with bovine alkaline phosphatase (Mesbah *et al.*, 1989). Deoxyribonucleosides were analysed by HPLC (LKB) on a Nucleosil 100-5 C18 (250 × 4 mm) column fitted with a guard column (20 × 4 mm) of the same material using the conditions described by Tamaoka & Komagata (1984). DNA-DNA hybridization was carried out according to the method of De Ley *et al.* (1970), with modifications, using a Gilford System 2600 spectro-

photometer equipped with a Gilford 2527-R thermo-programmer and plotter. The DNA concentration was 45 µg ml⁻¹. Due to the high G + C content of the DNA, 20% DMSO was added to 2.5 × SSC buffer to depress the DNA melting point (T_m) (Escara & Hutton, 1980). The resulting buffer was 2 × SSC + 20% DMSO. The mean T_m was determined from the DNA melting curves and the optimal temperature of renaturation (TOR) was calculated according to the equation $TOR = T_m - 25$.

The DNA-DNA hybridization values were calculated by regression analysis of the renaturation rates over the time period 10–30 min after starting the reaction using the computer program TRANSFER.BAS (Jahnke, 1992).

Host tests. The relative susceptibility of white and brown strains of *A. bisporus* to the soft rot disease was determined in crops grown using standard commercial procedures in two mushroom-growing rooms. Mushrooms were grown in pots (25 cm diameter) containing 2.5 kg fresh weight compost in a random block design. The eight treatments consisted of all combinations of brown (Sinden Hauser 3.8) or white (Horst U3) mushrooms, high (95%) or low (85%) relative humidity (RH), and with or without soft rot bacteria (W1r3^T) being added (24 replicates per treatment). An overnight culture of W1r3^T in King's B Broth (King *et al.*, 1954) was diluted with deionized water to 3.08 × 10⁷ c.f.u. ml⁻¹ and 100 ml was applied to half of the pots as a drench. Both rooms were maintained at 90% RH until mushroom fruit bodies initially appeared on the casing surface after 12 d. One chamber was then adjusted to 85% RH and the other to 95% RH. Mushrooms were harvested over 5 weeks and the weights and numbers which were healthy or diseased were recorded for each pot.

An *in vitro* experiment was performed to investigate the effect of RH on the development of disease symptoms. Sealed jars containing saturated solutions of salts were used as a simple method to provide atmospheres of known

humidities (ASTM, 1963; Young, 1967). Kilner jars (1 lb) contained 30 g of the relevant salt to which 30 ml sterile distilled water was added. White mushrooms (Horst U3), weighing approximately 8 g, were then placed in weighing boats, the jars were sealed with a metal screw on the lids, with a central stoppered inoculation port, and equilibrated at 18 °C for 2 h. The RH was then measured using a humidity probe (Vaisala) introduced through the inoculation port, connected to a data logger (SQ32; Grant Instruments). The mushrooms were inoculated by streaking strains W1r3^T, C1b4 and H5 onto the cap surfaces and then incubated for 4 d at 18 °C. The RH was checked every 24 h. The salts used and the RH achieved (expected RH in parentheses) at 18 °C were as follows: NaCl, 67.5 (76); (NH₄)₂SO₄, 71.5 (81); KCl, 75 (86); (NH₄)H₂PO₄, 80 (93); K₂SO₄, 85 (96.5); sterile distilled H₂O, 93 (100).

RESULTS

Bacterial colonies of the three strains (W1r3^T, C1b4 and H5) isolated from diseased mushrooms, grown on PAF after 48 h at 25 °C were 3–4 mm in diameter, a

translucent buff colour, circular, slightly convex, smooth and slightly mucoid. The texture was viscous. No pigment was produced initially but in older cultures, a buff, non-fluorescent pigment was seen. Strains were motile rods, 1.0–1.5 × 1.8–2.5 µm, occurring singly or in pairs. All strains were oxidase- and catalase-positive, but indole- and Voges–Proskauer-negative. Acid was produced from carbohydrates. No white line was produced between the test isolates and ‘*Pseudomonas reactans*’, indicating they were not *P. tolaasii* strains, and they did not grow well on PIA. Although a range of standard bacteriological tests were carried out with the isolate W1r3^T (data not shown), a positive identification could not be made from these results and a more detailed study was performed.

Isolates W1r3^T, C1b4 and H5 all produced disease symptoms when applied to mushroom tissue blocks. *J. lividum* NCIMB 9230 and 9414 produced slight necrosis. However, the type strain of *J. lividum*, DSM

Fig. 1. Soft rot of *A. bisporus* (Horst U3) caused by isolate W1r3^T. Bar, 1 cm.

Table 2. 16S rDNA sequence similarity values of strain W1r3^T and members of the genera *Janthinobacterium*, *Herbaspirillum*, *Duganella*, *Oxalobacter* and *Telluria* and *Pseudomonas lemoignei*

Strains: 1, W1r3^T; 2, *Janthinobacterium lividum* DSM 1522^T; 3, *Herbaspirillum seropedicae*; 4, *Duganella zoogloeoides*; 5, *Pseudomonas lemoignei*; 6, *Oxalobacter formigenes*; 7, *Telluria mixta*; 8, *Telluria chitinolytica*.

Strain	1	2	3	4	5	6	7	8
1	100.0							
2	99.0	100.0						
3	96.4	95.8	100.0					
4	96.1	96.5	95.0	100.0				
5	94.7	94.4	95.6	93.8	100.0			
6	93.2	93.1	95.4	92.7	93.3	100.0		
7	94.4	94.5	92.4	92.9	92.3	91.1	100.0	
8	92.9	93.0	92.7	93.5	91.4	90.6	96.2	100.0

Table 3. Fatty acid composition of members of the genus *Janthinobacterium*

Method 1 results in the release of ester-linked fatty acids; method 2 allows the detection of components which are presumptively amide-linked (methods were carried out at the different institutions given in the text). Numbers given are the percentages of each fatty acid compared to the total whole-cell fatty acids; TR, trace amount.

Fatty acid	Mushroom isolate						<i>Janthinobacterium lividum</i>							
	Strain ...		H5		C1b4		W1r3 ^T		DSM 1522 ^T		NCIMB 9414		NCIMB 9230	
	Method ...	1	2	1	2	1	2	1	2	1	2	1	2	
3-OH 10:0		2.48	3.38	2.38	3.16	2.13	3.20	2.39	4.03	1.69	3.57	2.64	4.03	
12:0		4.65	4.08	4.42	3.70	4.06	3.63	6.37	5.20	5.60	4.88	6.16	5.11	
2-OH 12:0		1.02	0.81	1.09	0.94	0.85	0.87	TR	TR	TR	TR	TR	TR	
14:0		1.57	1.44	1.42	1.29	1.26	1.22	1.14	1.05	1.39	1.22	1.15	1.07	
15:0		0.73	0.71							1.11	1.06		0.67	
16:1 ω 7cis		24.68	24.59	26.09	24.22	23.23	22.40	41.00	38.37	39.93	37.61	43.09	40.72	
16:0		38.65	40.92	38.20	40.35	38.93	40.98	33.02	36.11	34.24	36.17	33.95	35.33	
Unknown									0.72					
Cyclo-17:0		23.89	22.70	24.37	23.68	26.79	26.07	13.42	11.07	13.29	12.52	10.22	9.82	
18:1 ω 7cis		1.58	1.38	2.03	2.66	1.79	1.63	2.67	3.45	2.75	2.97	2.78	3.24	
% of 3-OH fatty acids which are potentially amide-linked			26.67		24.82		33.31		40.83		52.73		34.53	

Table 4. Fatty acid composition of members of the genus *Herbaspirillum*

Method 1 results in the release of ester-linked fatty acids; method 2 allows the detection of components which are presumptively amide-linked (methods were carried out at the different institutions given in the text). Numbers given are the percentages of each fatty acid compared to the total whole-cell fatty acids.

Fatty acid	<i>H. rubrisubalbicans</i> DSM 9440 ^T		<i>H. seropedicae</i> DSM 6445 ^T		<i>Herbaspirillum</i> sp. LMG 2285		<i>Herbaspirillum</i> sp. LMG 5321		<i>Herbaspirillum</i> sp. LMG 5523	
Method...	1	2	1	2	1	2	1	2	1	2
10:0					0.87					0.38
3-OH 10:0	1.39	1.45	0.85	1.05	0.78	1.14	0.70	0.80	1.13	1.23
12:0	4.33	3.36			3.23	2.98	4.35	4.18	2.97	2.92
2-OH 12:0			1.26	1.19						0.36
3-OH 12:0	1.33	2.93	0.97	2.72	0.65	2.62	1.90	3.71	0.85	2.61
14:0	0.96	0.83	3.74	3.56			0.71	0.67	0.55	0.51
2-OH 14:0	2.79	2.15	1.12	1.01	2.16	2.19	3.16	2.88	1.97	2.09
16:1 ω 7 cis	35.01	32.58	37.10	36.13	34.04	33.72	41.48	39.72	32.01	34.55
16:1 (?)	4.65	3.86	2.01	2.02	2.14	2.10	2.19	1.94	2.67	3.32
Unknown										0.52
16:0	22.13	23.66	22.71	23.06	20.75	22.49	18.66	19.89	18.34	21.15
Cyclo-17:0	6.22	5.92	4.40	4.12	7.09	6.34	3.24	2.96	3.04	3.29
Unknown	1.09		0.63	0.62		0.62			0.47	0.52
18:1 ω 7 cis	16.73	19.44	21.59	22.32	21.18	23.19	21.19	21.22	19.72	23.28
18:1 (?)	2.43	2.72	1.11	1.14	1.39	1.55	1.21	0.89	1.67	2.01
18:0	0.93	1.10	0.97	1.06	0.89	1.07	1.20	1.14	1.14	1.27
% of 3-OH fatty acids which are potentially amide-linked		37.97		51.55		62.10		42.19		48.45

1522^T, did not have any effect on the mushroom tissue after 48 h. When isolates W1r3^T or C1b4 were applied to beds of growing mushrooms, as a suspension in water, the disease symptoms could be reproduced and the bacteria re-isolated, thus satisfying Koch's postulates (Fig. 1). No soft rot symptoms were observed on control beds drenched with water, to which the test organisms had not been added, in the initial trial.

Chemical and molecular analysis

Fatty acid analysis of strains W1r3^T, C1b4 and H5 was initially carried out at CSL, Harpenden, and indicated that the fatty acid composition of these strains was

similar to that of *J. lividum*. Based on this knowledge, it was possible to test the hypothesis that these strains were related to *J. lividum*, although members of this genus have not been reported to be pathogenic for mushrooms previously. Full 16S rDNA sequences (1469 nt) were determined for isolate W1r3^T, a representative of the mushroom pathogen strains (*Escherichia coli* positions 29–1500). Analysis of the 16S rDNA sequence of strain W1r3^T showed that this organism was part of the β -subclass of the *Proteobacteria* (Woese, 1987). The 16S rDNA sequence of the type strain of *J. lividum* (DSM 1522^T) showed 99.0% (Table 2) sequence similarity to that of strain W1r3^T (EMBL no. Y08845), which is also consistent

Table 5. Percentage fatty acid composition of members of the genera *Janthinobacterium*, *Duganella*, *Herbaspirillum* and *Oxalobacter* and *Pseudomonas lemoignei*

Fatty acid	<i>Duganella</i> *	<i>P. lemoignei</i> †	<i>Oxalobacter</i> ‡	<i>Janthinobacterium</i>	<i>Herbaspirillum</i>
10:0	2	—	—	—	0.0–0.9
3-OH 10:0	+	3.6	—	3.1–4.0	0.8–1.4
12:0	4	5.3	0–1	3.6–5.2	0.0–4.1
2-OH 12:0	—	—	—	TR–0.94	0.0–1.1
3-OH 12:0	—	5.7	4–6	—	2.6–3.7
14:0	3	1.3	1–2	1.0–1.4	0.0–3.5
2-OH 14:0	+	4.7	8–10	—	1.0–2.8
16:1 ω 7 cis	41§	39.3	TR–1§	22.4–40.7	32.6–39.7
16:1 (?)	—	—	—	—	1.9–3.8
16:0	38	12.8	28–33	35.3–40.9	19.8–23.7
Cyclo-17:0	—	1.9	3–34	9.8–26.7	2.9–5.9
18:1 ω 7 cis	12§	25.5	TR–13§	1.38–3.45	19.4–23.2
18:1 (?)	—	—	—	—	0.8–2.7
18:0	—	—	TR–5	—	—
Cyclo-19:0	—	—	14–38	—	—

TR, Trace amount.

* Data from Hiraishi *et al.* (1992).† Data from Mergaert *et al.* (1996).‡ Data from Allison *et al.* (1985); includes both sub-groups.§ Hiraishi *et al.* (1992) and Allison *et al.* (1985) do not distinguish which isomers of 16:1 and 18:1 were present.

with the placement of *J. lividum* in this phyletic group in earlier reports based on DNA–RNA hybridization data (De Ley *et al.*, 1978). Although DNA–RNA hybridization data have indicated an evolutionary relationship between members of the genera *Janthinobacterium* and *Herbaspirillum* (Baldani *et al.*, 1996), the 16S rDNA sequence data showed that the organisms having the greatest degree of sequence similarity to the two *Janthinobacterium* species were members of the genera *Herbaspirillum*, *Oxalobacter* and *Duganella* and *P. lemoignei* (similarity values within the cluster >95%). The species *T. mixta* and *T. chitinolytica* were specifically associated with this phyletic group, but showed lower similarity values (<95%). Members of the genera *Ralstonia* and *Burkholderia* were the next closest, but distinct, phyletic group, showing less than 92% sequence similarity (data not shown). Having established that all these organisms belonged to the same phyletic group, a chemotaxonomic study was undertaken to establish to what degree these various genera were justified. Fatty acid analysis of the organism, together with respiratory lipoquinone analysis was particularly useful due to the application of this method to members of the genera *Oxalobacter* (Allison *et al.*, 1985) and *Duganella* (Hiraishi *et al.*, 1992, 1997), together with data for various strains of *P. lemoignei* (Mergaert *et al.*, 1996). The results obtained for the fatty acid composition of the *Janthinobacterium* and *Herbaspirillum* species used in this study (Tables 3 and 4) indicated that a clear separation could be made between the genus *Janthinobacterium* and the other

organisms for which data are already available (Table 5).

Analysis of the respiratory lipoquinone content of the mushroom pathogen and members of the genera *Janthinobacterium* and *Herbaspirillum* indicated that only ubiquinones were present and that the major component was Q-8 in all strains tested.

The polar lipid compositions of the mushroom pathogens and several strains of *J. lividum* were determined to test their assignment to the same genus. The polar lipids were essentially identical, comprising mainly phosphatidyl ethanolamine, phosphatidyl glycerol and diphosphatidyl glycerol (Fig. 2).

In view of the high degree of similarity at the 16S rDNA sequence and chemical levels, all further studies on the physiology of the mushroom pathogens were, therefore, restricted to a comparison with strains of the species *J. lividum*. The biochemical properties of the mushroom pathogen and several strains of *J. lividum* showed that two groups could easily be distinguished on the basis of the API 20NE, API 50CH and BIOLOG data (Tables 6, 7 and 8). In summary, strains of the mushroom pathogen were rather less reactive than strains of *J. lividum* tested. On the basis of these findings, using representative strains of the two groups, it was tested whether DNA–DNA hybridization would also support the placement of the two groups into different species, within the genus *Janthinobacterium*. DNA–DNA hybridization between *J. lividum* DSM

Fig. 2. Two-dimensional TLC of the polar lipids of *J. lividum* DSM 1522^T and *J. agaricidamnorum* W1r3^T (=DSM 9628^T). For details, see Methods. PG, Phosphatidyl glycerol; PE, phosphatidyl ethanolamine; DPG, diphosphatidyl glycerol; PL, phospholipid (not further characterized); PN, aminophospholipid (not further characterized).

1522^T and strain W1r3^T gave a value of 35% which is well below the value generally considered to identify isolates as the same species (Johnson, 1984).

The G + C content of the DNA was determined as 64.2 mol% (T_m method) for W1r3^T, which is within the range given for the genus *Janthinobacterium* (61–67 mol%), which is currently represented by a single species, *J. lividum* (Sneath, 1984).

The resistance of W1r3^T, C1b4, H5, NCIMB 9230, NCIMB 9414 and DSM 1522^T to a range of antibiotics was compared. All isolates were resistant to penicillin G (10 µg disc) and vancomycin (30 µg disc), but showed no resistance to erythromycin (15 µg disc),

streptomycin (10 µg disc), tetracycline (30 µg disc) or nalidixic acid (30 µg disc).

Many of the results obtained in this study are characteristic for *J. lividum* but the isolates obtained in this study do not produce the purple pigment usually associated with this species. This purple pigment, violacein, is produced from tryptophan and can be synthesized by *J. lividum*, *Chromobacterium violaceum*, *Iodobacter fluviatilis* and some members of the genus *Pseudoalteromonas*. However, it is possible to isolate and culture *J. lividum* and *C. violaceum* strains in a non-pigmented state (Logan & Moss, 1992; Shivaji *et al.*, 1991; Sivendra *et al.*, 1975). Often pigmented and non-pigmented colony forms were seen in the same culture of the strains obtained from NCIMB and sometimes the pigment was lost altogether for one or two subcultures. This is possibly an example of intracolonial polymorphism as encountered in fluorescent pseudomonads and other bacteria described by Rainey *et al.* (1992b, 1993). However, pigment was never seen in any of the isolates from mushrooms.

Host tests

Mushroom crop experiment. At 85% RH, the brown mushroom strains appeared to be affected less by the disease than the white strains. However, 95% RH had more of an effect on the disease on the brown strains than on the white strains, making them more susceptible to soft rot at the higher humidity. The rather high percentage of mushrooms affected without having bacteria added was probably due to contamination during picking, watering or cross-contamination by flies. The percentages of diseased mushrooms produced for each treatment are given in Table 9. Infected mushrooms were too degraded to weigh.

It is normal mushroom-growing practice to keep the RH in cropping houses at 90% (Hermans, 1988). However, W1r3^T was isolated from a farm where the disease was causing a problem at that humidity. By reducing the RH in a cropping house to below 85% by careful control of ventilation and watering, a loss in total yield may be experienced, but the loss of mushrooms to this disease may be restricted, in the absence of any additional chemical controls. This method is already used for controlling outbreaks of brown blotch disease, caused by the bacterium *P. tolaasii* (Fletcher *et al.*, 1989).

Laboratory humidity tests. The use of saturated salt solutions to create an atmosphere of known RH was partially successful. It was possible to equilibrate RH in sealed jars containing harvested mushrooms; however, the RH was lower than expected for each respective saturated salt solution. This was probably due to the nature of mushroom tissue, being absorbent and containing more than 90% (w/w) water. However, a general trend showed no disease symptoms on W1r3^T-inoculated mushrooms below 71.5% RH.

Table 6. Differential characteristics of species of the genus *Janthinobacterium* based on respiratory activity mediated using BIOLOG GN plates

Tests negative for all strains were: α -cyclodextrin, glycogen, *N*-acetyl-D-glucosamine, adonitol, D-arabitol, cellobiose, i-erythritol, α -D-lactose, D-melibiose, methyl β -D-glucoside, monomethylsuccinate, *cis*-aconitic acid, citric acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, α -hydrobutyric acid, γ -hydrobutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, quinic acid, D-saccharic acid, sebacic acid, glucuronamide, L-histidine, hydroxyl-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-pyrogutamic acid, DL-carnitine, γ -aminobutyric acid, uridine, thymidine, phenylethylamine, putrescine, 2-amino-ethanol and 2,3-butanediol. Tests positive for all strains were: α -D-glucose, sucrose, methylpyruvate, formic acid, β -hydrobutyric acid, bromosuccinic acid, alaninamide, L-alanyl-glycine, glycyl-L-aspartic acid, glycyl-L-glutamic acid, DL-glycerol phosphate, glucose 1-phosphate and glucose 6-phosphate. v, Variable within strains.

Test	<i>J. lividum</i>	<i>J. agaricidamnorum</i>
Gentiobiose	—	v
Lactulose	—	v
d-Trehalose	—	+
Turanose	—	v
D-Glucuronic acid	—	v
<i>m</i> -Inositol	—	v
Urocanic acid	—	v
Dextrin	+	—
<i>N</i> -Acetyl-D-galactosamine	+	—
D-Fucose	+	v
Maltose	+	—
D-Mannose	+	—
D-Sorbitol	+	v
Xylitol	+	—
DL-Lactic acid	+	—
Propionic acid	+	v
Succinic acid	+	—
Succinamic acid	+	—
D-Alanine	+	—
L-Alanine	+	—
L-Asparagine	+	—
L-Aspartic acid	+	—
L-Glutamic acid	+	—
L-Proline	+	—
Inosine	+	—
Tween 40	v	+
Tween 80	v	+
L-Arabinose	v	—
L-Fucose	v	—
D-Galactose	v	—
D-Mannitol	v	—
D-Psicose	v	—
D-Raffinose	v	—
L-Rhamnose	v	—
Acetic acid	v	v
<i>p</i> -Hydroxyphenylacetic acid	v	—
α -Ketobutyric acid	v	+
α -Ketoglutaric acid	v	—
α -Ketovaleric acid	v	+
Malonic acid	v	v
D-Serine	v	—
L-Serine	v	—
L-Threonine	v	—
Glycerol	v	+

Table 7. Differential characteristics of species of the genus *Janthinobacterium* based on carbohydrate utilization in API 50CH

Tests negative for all strains were: erythritol, L-xylose, adonitol, methyl β -xyloside, L-sorbose, dulcitol, methyl α -D-mannoside, methyl α -D-glucoside, amygdalin, aesculin, lactose, melibiose, melezitose, amido, glycogen, D-turanose, D-tagatose, D-fucose, L-arabitol, gluconate and 5-ketogluconate. Tests positive for all strains were: glycerol, ribose, D-glucose, D-fructose, D-mannose, inositol, mannitol, saccharose and D-arabitol. v, Variable within strains.

Test	<i>J. lividum</i>	<i>J. agaricidamnorum</i>
Trehalose	—	+
β -Gentiobiose	—	+
D-Arabinose	+	—
L-Arabinose	+	—
D-Xylose	+	—
Galactose	+	—
Sorbitol	+	—
Arbutin	+	—
Salicin	+	—
Cellobiose	+	—
Maltose	+	—
Xylitol	+	—
D-Lyxose	+	—
L-Fucose	+	—
2-Ketogluconate	+	—
Rhamnose	v	—
N-Acetylglucosamine	v	—
Inulin	v	—
D-Raffinose	v	—

Table 8. Differential characteristics of species of the genus *Janthinobacterium* based on API 20NE tests

Tests negative for all strains were: production of gas from nitrate, production of indole from tryptophan, fermentation of glucose, presence of arginine dihydrolase, presence of urease, β -galactosidase activity, assimilation of glucose, gelatin hydrolysis and assimilation of gluconate. Tests positive for all strains were: assimilation of glucose, assimilation of malate, assimilation of mannose and assimilation of mannitol. v, Variable within strains.

Test	<i>J. lividum</i>	<i>J. agaricidamnorum</i>
Nitrate reduction	+	—
Aesculin hydrolysis	+	—
Assimilation of:		
Arabinose	+	—
Maltose	+	—
Citrate	+	v
Phenylacetate	+	—
N-Acetylglucosamine	v	—
Caprate	v	—
Adipate	v	—

Slight pitting and blotching occurred at 80% RH. At 85% RH, the mushrooms showed the first typical soft rot symptoms and extensive rotting occurred at 95% RH.

DISCUSSION

The causal organism of this soft rot disease of *A. bisporus* is an aerobic Gram-negative rod which has a high degree of 16S rDNA sequence similarity to members of the genus *Janthinobacterium*. It appears to be most active under very moist conditions and thus may have been introduced to mushroom farms through the water supply or casing peat as *J. lividum* is known to be found in soil and water (Moss *et al.*, 1978; Moss & Ryall, 1981). Strains H5, W1r3^T and C1b4 and the three strains of *J. lividum*, including the type strain, were qualitatively identical in their chemical composition, with the exception of a 15:0 fatty acid present as a minor component in strains H5, NCIMB 9414 and 9230 only. However, there were biochemical differences between the type strain and two additional strains of *J. lividum* and the strains C1b4, H5 and W1r3^T. Based on these data, it was concluded that strains C1b4, H5 and W1r3^T should be placed in the genus *Janthinobacterium*, but as a separate species, a conclusion supported by the DNA-DNA hybridization data. Despite the fact that there is a high degree of 16S rDNA sequence similarity (>95%) to members of the genera *Herbaspirillum*, *Oxalobacter* and *Duganella* and *P. lemoignei*, these taxa are distinguishable on the basis of their fatty acid patterns from members of the genus *Janthinobacterium*. In particular, all organisms examined in this study and that of Mergaert *et al.* (1996) contained 16:0, 16:1 ω 7c (cyclo-17:0) and 18:1 ω 7c as the major fatty acids. Cyclopropane 17:0 fatty acids are synthesized from the corresponding 16:1 fatty acid and may be considered to be equivalent in the taxonomic evaluation. All organisms contained hydroxy fatty acids. However, in members of the genus *Janthinobacterium*, only 3-OH 10:0 and 2-OH 12:0 were synthesized, whereas in members of the genus *Herbaspirillum* and *P. lemoignei*, the major hydroxy fatty acids present were 3-OH 10:0, 3-OH 12:0 and 2-OH 14:0. *Duganella zoogloeoides* has been shown to synthesize only 3-OH 10:0 and 2-OH 14:0, whereas *Oxalobacter formigenes* produces 3-OH 12:0 and 2-OH 14:0. Examination of the distribution of ester- and (presumptive) amide-linked 3-OH fatty acids indicated that in members of the genus *Herbaspirillum* the 3-OH 10:0 and 2-OH 14:0 fatty acids were ester-linked, whereas the 3-OH 12:0 fatty acid was ester- and (presumptively) amide-linked. In contrast, the 3-OH 10:0 fatty acid of members of the genus *Janthinobacterium* were ester- and (presumptively) amide-linked, and the 2-OH 12:0 fatty acids were ester-linked. Data on the distribution of ester- and amide-linked fatty acids from the other organisms is lacking in the literature. An interesting feature is that the use of differential hydrolysis of the fatty acids adds another dimension to the use of fatty

Table 9. Effect of RH in cropping house and mushroom spawn strain on expression of W1r3^T soft rot disease symptoms

Mushroom strain	RH (%)	+ W1r3 ^T *	Control*
Brown (Sinden Hauser 3.8)	95	66.26	33.21
	85	23.21	1.79
White (Horst U3)	95	54.61	29.49
	85	35.76	2.64

* Percentage diseased mushrooms by number; 24 replicated; standard error of differences 3.95.

acid analysis in prokaryotes. The fatty acid composition of the phyletic group defined by the genera *Burkholderia* and *Ralstonia* is also different, there being significant amounts of 2-OH 16:0 and 3-OH 14:0 fatty acids. In addition, some species may also contain 2-OH 16:0, 2-OH 16:1, 2-OH 18:1 and 3-OH 16:0, the latter being amide-linked in the polar lipid fraction (Cox & Wilkinson, 1989; Galbraith & Wilkinson, 1991; Oyaizu & Komagata, 1983; Yabuuchi *et al.*, 1992, 1995; Stead, 1992), which clearly differentiates the *Burkholderia*–*Ralstonia* phyletic group from the *Janthinobacterium*–*Duganella*–*Oxalobacter*–*Herbaspirillum* phyletic group. On the basis of the fatty acid data available, we consider that members of the genus *Janthinobacterium* can be distinguished from members of the genera *Duganella*, *Oxalobacter* and *Herbaspirillum*, which we conclude supports the distinct taxonomic rank of these genera. This is consistent with the placement of the organisms within the β -subclass of the *Proteobacteria* and is also in accordance with the finding of Q-8 in members of the genera *Duganella* (Hiraishi *et al.*, 1992, 1997) and *Telluria* (Bowman *et al.*, 1993). There are other differences between the various other taxa, which would indicate that they also are deserving of placement in different genera. Members of the genus *Oxalobacter* are obligate anaerobes (Allison *et al.*, 1985), whereas members of the genus *Herbaspirillum* utilize a much wider range of substrates than members of the genus *Janthinobacterium* (Baldani *et al.*, 1996; B. J. Tindall, unpublished results). If one were to reduce this phyletic group to a single genus, for which the name *Janthinobacterium* has priority, the genus would comprise a physiologically and chemically heterogeneous assemblage. Although closer study of members of the genus *Herbaspirillum* and *P. lemoignei* should be undertaken, it is quite clear that each of the genera compared in the present study may be unambiguously distinguished from one another and we advocate retaining these genera. These results also indicate that the use of similarity values alone to define taxa as previously proposed should be questioned (Devereux *et al.*, 1990; Fry *et al.*, 1991), since it amounts to an overweighting of the 16S rDNA data, rather than a balanced integration with other data of evolutionary relevance. The arbitrary assignment of an organism to a higher taxonomic rank (i.e. genus)

should be supported by appropriate evidence. The problem has been discussed previously (Tindall, 1994). Although such guidelines (i.e. sequence similarity values) may be useful, they should be treated with caution and altered according to the state of our knowledge. In the present work, we have presented chemical evidence which clearly separates the genus *Janthinobacterium* from other genera with which it shares between 95 and 96.6% sequence similarity.

The results presented here may be taken as supporting the hypothesis that the mushroom pathogenic strains show a high degree of similarity and, therefore, constitute a monophyletic group, for which placement in the same species would be appropriate. They also share similarities with members of the genus *Janthinobacterium* (Tables 3, 6, 7 and 8) and members of this genus, in turn, share similarities with members of the genera *Herbaspirillum*, *Duganella* and *Oxalobacter* (Table 2). Despite the high degree of similarity, a number of phenotypic differences were noted between the strains of the species *J. lividum* tested and strains C1b4, H5 and W1r3^T. These differences may be used to distinguish between members of the species *J. lividum* and strains C1b4, H5 and W1r3^T, which may be placed in a new species within the genus *Janthinobacterium*, for which we propose the name *Janthinobacterium agaricidamnorum* sp. nov.

The current definition of the genus *Janthinobacterium*, based on the descriptions of De Ley *et al.* (1978) and Sneath (1984), would exclude the new species from this genus. It is, therefore, necessary to emend the description of this genus.

Janthinobacterium (De Ley *et al.*, 1978) emend.

Data of significance in defining members of the genus *Janthinobacterium* are given in Tables 3, 6, 7 and 8, based on fatty acid patterns and the API 20NE, API 50CH and the BIOLOG test systems, which allow the placement of a strain in the genus *Janthinobacterium* as emended in this work. The fatty acid pattern also serves to differentiate this genus from members of other genera showing a high (>95%) degree of sequence similarity (Table 5). Q-8 is the major respiratory lipoquinone, as in all members of the β -subclass of the *Proteobacteria* studied to date (Collins

& Jones, 1981; Yokota *et al.*, 1992; B. J. Tindall, unpublished results). The major phospholipids are phosphatidylethanolamine, phosphatidyl glycerol and diphosphatidyl glycerol. The fatty acid composition comprises 3-OH 10:0, 12:0, 2-OH 12:0, 14:0, 16:0, 16:1 ω 7c, cyclo-17:0 and 18:1 ω 7c. A significant fraction of the 3-OH 10:0 fatty acid is (presumptively) amide-linked.

Description of *Janthinobacterium agaricidamnosum* sp. nov.

Janthinobacterium agaricidamnosum (a'ga'ri.ci.dam.nosum. L. masc. n. *agaricus* mushroom; L. adj. *damnosum* damaging; *agaricidamnosum* damaging the mushroom).

Rods 1.0–1.5 $\times$ 1.8–2.5 μ m. Cells occur singly or in pairs. No resting stage is known. Cells stain Gram-negative with lipid inclusions. Motile. Produce low convex, round, beige colonies on solid media. Maximum growth temperature, 30 °C; minimum, 2 °C. No growth below pH 5 or in media containing 2.9% NaCl. Acid is produced from glucose. Oxidase-positive, catalase-positive, indole-negative and Voges-Proskauer-negative. Nitrate and nitrite are not reduced. Utilizes citrate and ammonium ions as sole carbon and nitrogen sources for growth. The organism does not produce a purple pigment. Resistant to penicillin G (10 μ g disc) and vancomycin (30 μ g disc). 16S rDNA sequence analysis of strain W1r3^T shows 99% similarity to the type strain of *Janthinobacterium lividum* (DSM 1522^T). The G+C content is 64.2 mol%. Chemical composition is as described for the genus. Tests which allow the rapid differentiation of *J. lividum* and *J. agaricidamnosum* are summarized in Tables 6, 7 and 8. The type strain (W1r3^T) was isolated as the cause of a soft rot disease of the cultivated mushroom *A. bisporus*. Strain W1r3^T has been deposited in culture collections as DSM 9628^T and NCPPB 3945^T.

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