SHORT COMMUNICATION

OCCURRENCE OF BACTERIAL SOFT ROT OF *PLEUROTUS OSTREATUS* CAUSED BY *BURKHOLDERIA GLADIOLI* pv. *AGARICICOLA* IN KOREA

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SUMMARY

An unknown bacterium was isolated from cultivated oyster mushrooms grown in Korea, which caused a soft rot that developed more rapidly than that elicited by the brown blotch bacterium, *Pseudomonas tolaasii* at temperatures above 25ºC. From these lesions a bacterial strain (designated OM1) was isolated which, following inoculation of mushroom caps, yielded characteristic sunken water-soaked brown spots, which developed into a severe soft rot. In the early stages, the lesions were indistinguishable from those of bacterial brown blotch, well known to mushroom growers. Results of Gram stain and biochemical tests identified the new bacterial isolate as *Burkholderia gladioli* pv. *agaricicola*. This was confirmed by pathogenicity to oyster mushroom, physiological and biochemical properties, analysis of the 16S rRNA gene sequences and the fatty acids profile. This is the first report of the isolation of *B. gladioli* pv. *agaricicola* from cultivated oyster mushroom in Korea.

Key words: bacterial soft rot, mushroom, *Pleurotus ostreatus*, *Burkholderia gladioli* pv. *agaricicola*, 16S rRNA gene, fatty acids

Mushrooms are cultivated as one of the major economically profitable crops in many areas of Korea. The production areas have steadily increased from approximately 3,674 ha in 2002 to 4,118 ha in 2005. Several bacteria are known as the causal agents of diseases of the cultivated button mushroom (*Agaricus bisporus*) and oyster mushroom (*Pleurotus ostreatus*) (O'Riordain, 1976; Wong *et al*., 1982). There are different bacterial diseases of cultivated mushroom such as brown blotch, mummy disease, bacterial pit, bacterial rot and weeping disease, ginger blotch, and drippy gill (Betterley and Olson, 1989; Paine, 1919; Wong *et al*., 1982; Young, 1970). Although *Pseudomonas tolaasii* is known as the causal agent of bacterial brown blotch (Tolaas, 1915; Paine, 1919), much controversy exists regarding its identification and whether brown blotch may be caused by more than one organism.

Burkholderia gladioli pv. *agaricicola* is an additional threat for the cultivated mushroom industry, as it causes soft rotting of a number of commercially important species, such as *Lentinula edodes, Pleurotus ostreatus, Flammulina velupies, Pholiota nameko, Hypsizygus marmoreus*, and *Grifola frondos* in Japan, and of different *Agaricus* species in New Zealand and Europe (Gill and Tsuneda, 1997; Fermor and Lincoln, 2001).

Soft rot disease by *B. gladioli* pv. *agaricicola* was first reported by Gill and Cole (1992). The causal microorganism was initially identified as *Pseudomonas cepacia* (Burkholder) Palleroni and Holmes (Palleroni, 1984; Gill and Cole, 1992) to be later reclassified as *B. gladioli* pv. *agaricicola* (Gill and Tsuneda, 1997).

Oyster mushrooms showing soft rot symptoms were observed in spring 2007 in a Korean mushroom farm. Small pale brown spots were first seen on very young sporophores and, as these increased in size, the affected area also increased so that, eventually, 10-20% of the surface area appeared sunken and brown (Fig. 1 A). To isolate the putative causal agent, approximately 1 g of infected mushrooms pileus was crushed in sterile 1.5 ml Eppendorf tubes containing 1 ml sterile distilled water. The crushed mushroom suspension was serially diluted, plated onto R2A agar (Kim and Whang, 2002) and incubated at 28ºC. Bacterial colonies were purified on R2A agar or King's B medium and maintained at -70ºC in Luria-Bertani medium (LB) (Difco Laboratories, USA) containing 15% (v/v) glycerol. Each isolate was 'white line tested' for identity with *P. tolaasii* (Wong and Preece, 1979) and tested on mushroom tissue blocks for an initial pathogenicity assay (Gandy, 1968).

Bacterial isolates producing severe symptoms were obtained from mushrooms growing in three different farms. In all cases, the isolates had the same colony morphology, produced disease symptoms identical to those of the mushrooms soft-rot isolate OM1, and showed 99% 16S rRNA sequence homology with that of *B. gladioli*. Therefore, we selected strain OM1 as a type culture for further studies.

Isolates grown for two days on King's B medium

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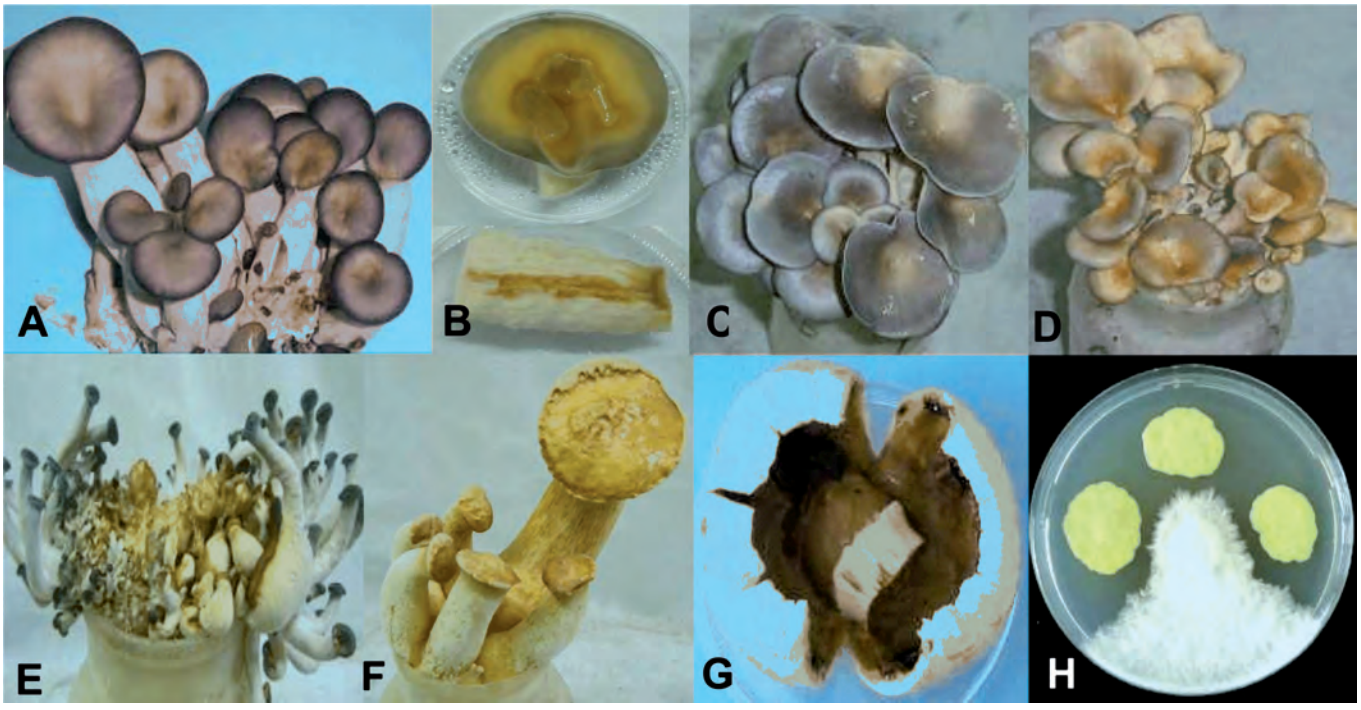

Fig. 1. A. Typical natural symptoms of bacterial soft rot on mushroom as observed in a mushroom cultivation farm. B. Tissue soft rot of mushroom inoculated by dropping a bacterial suspension on the sporocarp. C, D, E, F. Symptoms of bacterial soft rot on oyster and king oyster mushroom inoculated by spraying. G. Tissue soft rot on button mushroom injected with *B. gladioli* pv. *agaricicola*. H. Inhibition of oyster mushroom mycelium by *B. gladioli* pv. *agaricicola*.

were dispersed in sterile distilled water and used as inoculum for pathogenicity tests, after adjusting the concentration to 4×10^7 CFU/ml. Fresh oyster mushrooms and button mushrooms were washed twice with sterile distilled water, then the inoculum was transferred into pileus and stipe tissues with a sterilized needle or by dropping the suspension onto the cap. The inoculation was extended also to the sporocarps, by injecting a bacterial suspension, about 4×10^7 CFU/ml, into the fresh stipe of the white button mushroom. After inoculation, the mushrooms were incubated at ambient temperature (23-25°C) in a moist chamber for 3 days and examined for symptoms. Controls consisted of a batch of mushrooms treated with sterile distilled water only. Inoculations were also carried out on oyster and king oyster mushrooms growing in production rooms (16±2°C, RH>96%), by spraying a pure bacterial culture solution adjusted to *ca.* 4×10^7 CFU/ml.

Strain OM1 caused typical soft rot symptoms on the stipe and pileus of the oyster mushrooms two days after artificial inoculation by dropping (Fig.1 B). In production rooms, brown, water-soaked and very soft lesions developed on the sporocarps of oyster and king oyster mushrooms (Fig. 1C, D, E, F) which, with time, did spread all over the sporocarp causing tissue deterioration and necrosis (Fig. 1C, D). Because of these symptoms, we suggest the name "bacterial soft rot of oyster mushroom" for this disease. White button mushroom injected with a bacterial suspension showed a rapid soft watersoaking or deep brown discolouration of sporophore tissue within two days from inoculation (Fig. 1G). When oyster mushrooms were coincubated with *B. gladioli* pv. *agaricicola* mycelial growth was substantially inhibited (Fig. 1H). No symptoms were observed with the water control.

The pathogenicity of the bacterial isolate used for inoculation was thus established and Koch's postulates fulfilled. One typical isolate was deposited in the Korean Agricultural Culture Collection as KACC 13944 (originally indicated as strain OM1). The type species of *B. gladioli* pv. *agaricicola* NCPPB 3580, used for comparison, was obtained from National Collection of Plant Pathogenic Bacteria, York, UK.

Colony morphology was assessed on nutrient agar, 1% dextrose nutrient agar and King's B as described by Roberts (1973) and Lincoln and Fermor (1991). As seen under the electron microscope in 2% phosphotungstic acid mounts, bacterial cells were single or in pairs, rod-shaped with rounded ends, had an average size of 0.8-1.3×1.2-2.3 µm, and bore a single polar flagellum (not shown).

The following cultivation techniques and biochemical tests were employed for identifying the bacterium: Gram stain, presence of oxidase and gelatinase activity, litmus milk test, nitrate reduction, indole production, lipolytic activity, and acid production from different

Table 1. Comparison of physiological and biochemical characteristics of mushroom soft-rot isolate with type cultures of *B. gladioli* pv. *agaricicola.*

Characteristic	OM1	*B. gladioli* pv. *agaricicola* NCPPB 3580
No. of flagella	1-2	1-2
Gram reaction	-	-
Levan production	-	-
Diffusible pigments	+	+
Poly-β-hydroxybutyrate	+	+
Slime production from sucrose	+	+
Gelatine liquefaction	+	+
Lecithinase (egg yolk)	+	+
Lipase (Tween 80 hydrolysis)	+	+
Oxidase reaction	-	-
Arginine dihydrolase	-	-
Tobacco hydrolysis	+	+
Potato rot	+	+
Nitrate reduction	-	-
H2S production	-	-
Blotch test	-	-
White line	-	-
Growth at 4ºC	±[a]	±
Growth at 41ºC	-	-

OM1, mushroom soft-rot bacteria; [a]very slight growth at the bottom of the tube.

sugars, starch hydrolysis (Dowson, 1957) and production of pectinase on potato and carrot slices (Henniger, 1965). Carbon source utilization was studied using the compounds reported in Table 2, using a minimal medium with 0.1% (w/v) carbon source, according to Stanier *et al.* (1966). Additional biochemical properties and enzyme activities were assessed using the API 20E, API 20NE, API 50CH and API ZYM systems (bio-Mérieux). API ZYM tests were read after 4 h incubation at 37ºC while the outcome of the other tests was read after at least 48 h at 30ºC.

On 1% dextrose nutrient agar after three days, colonies were circular, 1.5-2.6 mm in diameter, flat convex, cream-coloured, semi-transparent with an entire edge, glistening and characteristically wrinkled with a diffusible, non-fluorescent yellow pigment. On nutrient agar the colonies were small, circular, convex, dirty white, flat semi-translucent and smooth with a greenish yellow zone, non-fluorescent, yellow diffusible pigment. On King's B agar after two days, the colonies were small, 1-2 mm in diameter yellow/green, circular, pulvinate, slightly viscid, and produced a diffusible non-fluorescent yellow pigment.

Isolate OM1 had an oxidative metabolism of glucose, was Gram-negative, arginine dehydrolase negative, blotch test and white line negative, able to liquefy gelatin and accumulate poly-β-hydroxybutyrate. It did not reduce nitrate and grew at 4ºC but not at 41ºC. It produced slime in mineral media containing 4% sucrose, gave a positive egg-yolk reaction and hydrolized Tween 80 but did not fluoresce on KB medium. Indole and hydrogen sulphide were not produced but the lipolytic activity was high (Table 1). For carbon source was utilized as shown in Table 2. Starch was not hydrolyzed, but pectin (polygaracturonic acid, PGA; sodium salt) de-

Table 2. Key nutritional tests of value in mushroom soft-rot isolate with type cultures of *B. gladioli* pv. *agaricicola.*

Utilization of	OM1	*B. gladioli* pv. *agaricicola* NCPPB 3580	Utilization of	OM1	*B. gladioli* pv. *agaricicola* NCPPB 3580
Saccharose	-	-	creatine	-	-
xylitol	-	-	5-aminovalerate	-	-
D-fucose	+	+	3-aminobenzoate	-	-
acetamide	-	-	acetamide	-	-
glutamate	-	-	butylamine	-	-
nicotinate	±[b]	±	DL-norvaline	±	±
5-aminovalerate	-	-	benzylamine	-	-
saccharate	+	+	putrescine	-	-
citraconate	-	-	tryptamine	-	-
mesaconate	±	±	meso-erythritol	-	-
4-hydroxybenzoate	+	+	2,3-butanediol	-	-
DL-mandelate	-	-	n-amylamine	+	+
histamine	-	-	glycollate	-	-
terephthalate	±	±	urea	-	-
glycine	-	-	inulin	-	-
L-norleucine	-	-	Acid production from:		
DL-aminobytyrate	+	+	L-arabinose	+	+
D-tryptophan	-	-	D-galactose	+	+
L-citrulline	-	-	Fructose	+	+

OM1, mushroom soft-rot bacteria; [b]very slight growth at the bottom of the tube.

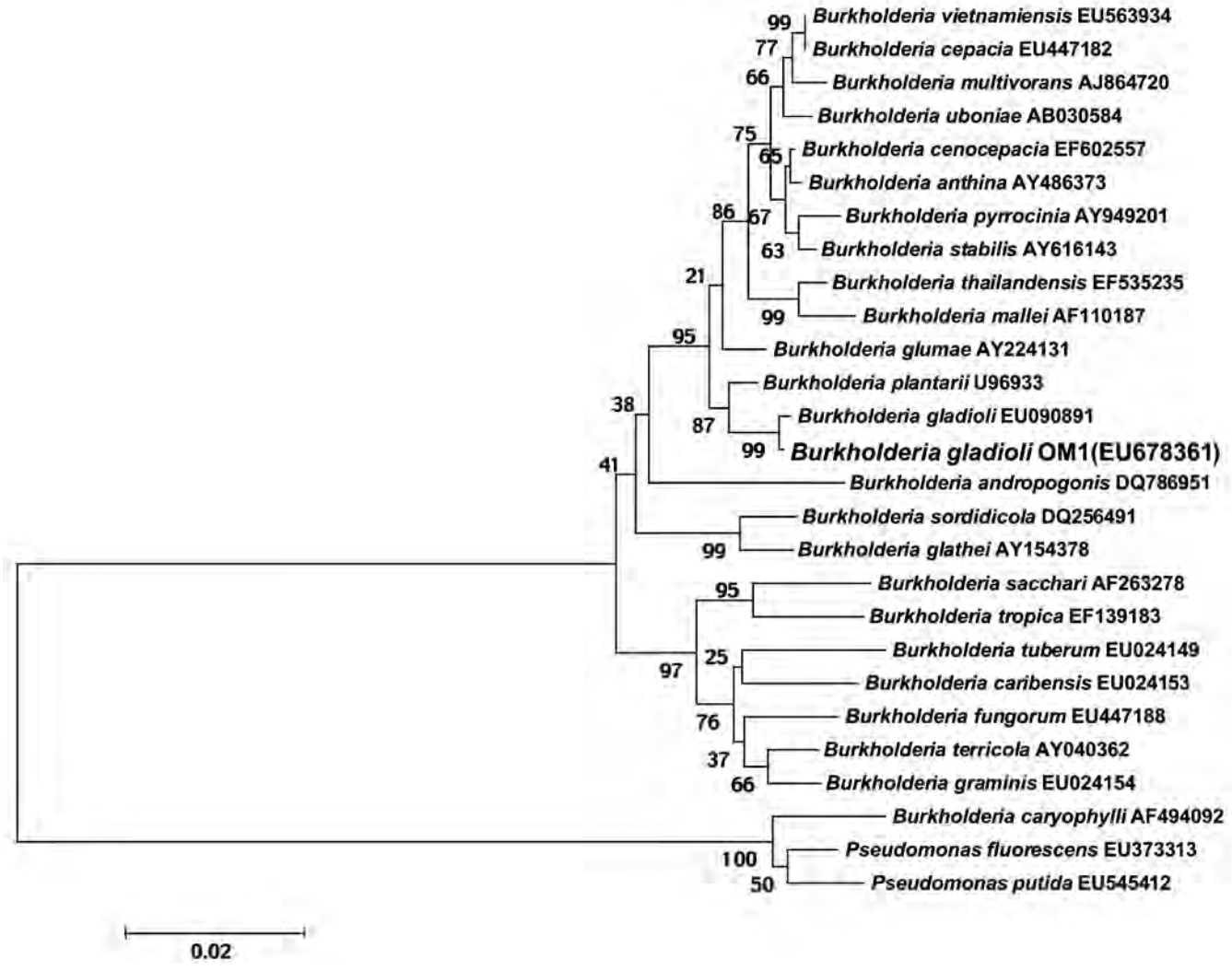

Fig. 2. Phylogenetic tree constructed using the neighbor-joining method, comparing the 16S rDNA sequences of strain OM1 from this study and members of the *Burkholderia* group from GenBank. Accession numbers are provided in parentheses. The bar represents a phylogenetic distance of 2%.

composition occurred at pH 5.0. OM-1 rotted onion slices and whole bulbs, and potato tuber slice at 25ºC (data not shown). In assays with the API ZYM system, esterase(C4), esterase lipase(C8), leucine arylamidase, acid phosphatase, valine arylamidase, lipase(C14), cystine arylamidase, β-glucosidase, α-mannosidase, naphthol-AS-BI-phosphohydrolase, N-acetyl-β-glucosaminidase were present, but trypsin, α-chymotrypsin, alkaline phosphatase, β-galactosidase, α-galactosidase, β-glucuronidase, α-glucosidase, α-fucosidase were not detected.

For fatty acid methyl ester analysis, cells were harvested from trypticase soy agar (TSA, Difco Laboratories, USA) plates after two days incubation at 30ºC. Fatty acids were extracted and their methyl esters were prepared in accordance with the standard protocol of the MIDI Hewlett-Packard Microbial Identification System (Sasser, 1990). The major fatty acid components of strain OM1 were 16:0 (18.0%), 18:1w7c (14.4%), sum in feature 3 (13.4%, comprising 16:1 w7c/15 iso 2OH), sum in feature 2 (12.5%, comprising 14:0 3OH/16:1 iso I), 17:0 cyclo (11.7%), 16:0 3OH (6.3%), 19:0 cyclo w8c (5.53%), 10:0 3OH (4.67%), 18:1 2OH (1.93%), 16:1 2OH (1.79%), and 16:0 2OH (0.81%). Lincoln *et al.* (1991) reported that the mushroom soft-rot strains are members of *Pseudomonas* rRNA group 2 and contain 16:0 3OH, 16:1 2OH, 16:0 2OH and 18:1 2OH (Oyaizu and Komagata, 1983; Palleroni, 1984). All mushroom soft-rot strains contained 10:0 3OH and produced a yellow pigment.

For the 16S DNA sequence analysis, DNA was isolated from pure bacterial cultures using the Qiagen Genomic DNA Isolation Kit (Qiagen, USA). PCR was carried out in a Peltier PTC-100 thermal cycler using the universal eubacterial primers fD1 [5´-AGAGTTTGATCCTGGCTCAG-3´, positions 7 to 26 in the *Escherichia coli* 16S rRNA gene (Brosius *et al.*, 1981)] and rP2 [5´-ACGGC-

TACCTTGTTACGACTT-3´, positions 1513 to 1494] (Weisburg *et al*., 1991). Sequencing and assembly of the 16S rRNA gene were determined by PCR amplification (Lane, 1991) and direct sequencing (Hiraishi, 1992).

The resulting 16S rRNA gene sequence (1400 nt) of strain OM1 was compared with 16S rRNA gene sequences available from GenBank using the BLAST program to determine the approximate phylogenetic affiliation, after which the gene sequence was then multiple aligned with those of closely related species by using the MEGALIGN program (DNASTAR). Phylogenetic trees were constructed using the neighbour-joining method (Saitou and Nei, 1987) with the MEGA3.0 program (Kumar *et al*., 2004); bootstrap percentages were based on 1000 replications (Felsenstein, 1985).

The 16S rRNA sequence of strain OM1 (GenBank accession No. EU678361) had 99% homology with that of *B. gladioli*. According to the inferred phylogenetic relationships derived from a neighbor-joining analysis, comparing the 16S rRNA gene sequence of OM1 with that of 24 validly described *Burkholderia* species isolate, OM1 was most closely related to the *B. gladioli* (Fig. 2).

In conclusion, OM-1 was definitely identified as *B. gladioli* by 16S rRNA gene sequences and fatty acid analysis, and as *B. gladioli* pv. *agaricicola* by morphological, biochemical and physiological characters (Lincoln *et al.,* 1991). In pathogenicity tests, this bacterium induced typical brown, water-soaked brown spots and soft rot. The lesions spread all over the sporocarp and cause tissue deterioration and necrosis. *B. gladioli* pv. *agaricicola* has been reported to cause soft rot disease on several mushroom species such as *Agaricus, Pleurotus* and *Flammulina* (Gill and Tsuneda, 1997). Lincoln *et al*. (1991) reported the isolation and characterization of a pseudomonad responsible for causing soft rot symptom on *Agaricus bitoquis* in the UK. This non-fluorescent pseudomonad was the first reported member of *Pseudomonas* rRNA group 2 (Palleroni, 1984) to be responsible for any fungal disorder, and they proposed it as a new pathovar, *Pseudomonas gladioli* Severini pv. *agaricicola*. Meanwhile, *Pseudomonas gladioli* Burkholder was reclassified as *Burkholedria gladioli* by Yabuuchi *et al.* (1992). This is the first documented record of soft rot of oyster mushroom caused by *B. gladioli* pv. *agaricicola* in Korea although the pathogen has been consistently isolated since 2007.

REFERENCES

Betterley D.A., Olson J.A., 1989. Isolation, characterization and studies of bacterial Mummy disease of *Agaricus brunnescens*. *Mushroom Science* **121**: 679-688.

Brosius J., Dull T.J., Sleeter D., Noller H.F., 1981. Gene organisation and primary structure of a ribosomal DNA operon from *Escherichia coli*. *Journal of Molecular Biology* **148**: 107-127.

Dowson W.J., 1957. Plant Diseases due to Bacteria. Cambridge University Press, London, UK.

Felsenstein J., 1985. Confidence limits on phylogenies: an approach using the booststrap. *Evolution* **393**: 783-791.

Fermor T., Lincoln S., 2001. Rotten mushrooms. International Society for Mushroom Science. http://www.isms.biz/article 13.htm.

Gandy D.E., 1968. A technique for screening bacteria causing Brown blotch of cultivated mushrooms. *Annual Report of the Glasshouse Crops Research Institute 1968*: 150-154.

Gill W.M., Cole A.L.J., 1992. Cavity disease of *Agaricus bitorquis* caused by *Pseudomonas cepacia*. *Canadian Journal of Microbiology* **383**: 394-397.

Gill W.M., Tsuneda A., 1997. The interaction of soft rot bacterium *Pseudomonas gladioli* pv. *agaricicola* with Japanese cultivated mushrooms. *Canadian Journal of Microbiology* **43**: 639–648.

Henniger H., 1965. Untersuchungen über Knollen- und Lagerfäulen der kartoffel. I. Zur Methodik der Resistenzprüfung mit Erreger der bakteriellen Knollennaßfäule (*Pectobacterium carotovorum* var. *Atrosepticum* (van Hall) Dowson). *Züchter* **353**: 174-180.

Hiraishi A., 1992. Direct automated sequencing of 16S rDNA amplified by polymerase chain reaction from bacterial cultures without DNA purification. *Letters in Applied Microbiology* **151**: 210-213.

Kim I.G., Whang K.S., 2002. The observation and a quantitative evaluation of viable but non-culturable bacteria in potable groundwater using epifluorescence microscopy. *The Korean Journal of Microbiology* **383**: 180-185.

Kumar S., Tamura K., Nei M., 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinformatics* **5**: 150-163.

Lane D.J., 1991. 16S/23S rRNA sequencing. In: Stackebrandt E., Goodfellow M. (eds). Nucleic Acid Techniques in Bacterial Systematics, pp. 115-175. Wiley, Chichester, UK.

Lincoln S.P., Fermor T.R., Stead D.E., Sellwood J.E., 1991. Bacterial soft rot of *Agaricus bitorquis*. *Plant Pathology* **404**: 136-144.

O'Riordain F., 1976. A disease of cultivated mushrooms caused by *Pseudomonas agarici* Young. *Irish Journal of Agricultural and Food Research* **2**: 250.

Oyaizu H., Komagata K., 1983. Grouping of *Pseudomonas* species on the basis of cellular fatty acid composition and the quinone system with special reference to the existence of 3-hydroxy fatty acids. *Journal of General and Applied Microbiology* **292**: 17-40.

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

Robert P., 1973. New or uncommon plant disease and pests. *Plant Pathology* **222**: 98.

Saitou N., Nei M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**: 406-425.

Sasser M.J., 1990. Identification of bacteria by gas chromatography of cellular fatty acids. Technical note 101, Microbial ID Inc., Newark, Del. USA.

Stainer R.Y., Palleroni N.J., Doudoroff M., 1966. The aerobic pseuomonads: a taxonomic study. *Journal of General Microbiology* **434**: 159-271.

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

Weisburg W.G., Barns S.M., Pelletier D.A., Lane D.J., 1991. 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* **1731**: 697-703.

Wong W.C., Fletcher J.T., Unsworth B.A., Preece T.F., 1982. A note on ginger blotch, a new disease of the cultivated mushroom *Agaricus bisporus*. *Journal of Applied Bacteriology* **525**: 43-48.

Wong W.C., Preece T.F., 1979. Identification of *Pseudomonas tolaasii*: the white line in agar and mushroom tissue block rapid pitting test. *Journal of Applied Bacteriology* **47**: 401-407.

Yabuuchi E., Kosako Y., Oyaizu H., Yano I., Hotta H., Hashimoto Y., Ezaki T., Arakawa M., 1992. Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. *Microbiology and Immunology* **36**: 1251-1275.

Young J.M., 1970. Drippy gill: a bacterial disease of cultivated mushrooms caused by *Pseudomonas agarici* n. sp. *New Zealand Journal of Agricultural Research* **131**: 977-990.

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