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Occurrence of *Pseudomonas tolaasii* on fruiting bodies of *Lentinula edodes* formed on *Quercus* logs*

Akihiko Tsuneda[1], Kazuo Suyama[2], Shigeyuki Murakami[1] and Ikuo Ohira[1]

[1] Tottori Mycological Institute, Kokoge 211, Tottori 689-11, Japan
[2] Department of Agriculture, Tokyo University of Agriculture, Tokyo 156, Japan

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A bacterial disease occurred on fruiting bodies of *Lentinula edodes* that formed outdoors on *Quercus* bedlogs during winter. The pathogen was identified as *Pseudomonas tolaasii* based on morphological and bacteriological characteristics. Symptoms exhibited by infected fruiting bodies ranged from mild browning to severe necrotic cavities that characteristically developed in the cap tissue along the periphery of the attachment area to the stalk. The mode of symptom development was greatly influenced by the internal tissue structure of fruiting bodies. Multiplication of bacterial cells within the fruiting bodies was strictly intercellular and thus differed from previously reported bacterial disease of *L. edodes* incited by an unidentified rod-shaped bacterium. The present strain of *P. tolaasii* was capable of attacking the *L. edodes* mycelium in the inner bark and outer sapwood regions and caused lysis of heavily infected hyphae.

Key Words——bacterial disease; electron microscopy; *Lentinula edodes*; microbial interaction; *Pseudomonas tolaasii*.

Pseudomonas tolaasii Paine is well known as the cause of bacterial blotch of the button mushroom, *Agaricus brunnescens* Peck (=*A. bisporus* (Lange) Imbach) (Tolaas, 1915), and is responsible for considerable losses of mushrooms every year (Fermor, 1987). Its infection spreads under moist conditions and causes discoloration of infected mushrooms only when the bacterial population is very large (Fletcher et al., 1989). In Japan, diseases caused by *P. tolaasii* are widespread among edible mushrooms that are cultivated indoors using sterilized, nutrient-supplemented sawdust as culture medium (Suyama and Fujii, 1993). These include *Pleurotus ostreatus* (Jacq.: Fr.) Kummer (oyster mushroom), *Lentinula edodes* (Berk.) Pegler (shiitake mushroom), and *Flammulina velutipes* (Curt.: Fr.) Sing. (winter mushroom). We have long considered this bacterium to be a possible threat also to the outdoor cultivation of *L. edodes*, and in fact, we recently isolated *P. tolaasii* from diseased fruiting bodies developed on *Quercus* bedlogs in two prefectures, Iwate and Mie. We report herein some bacteriological characteristics of the pathogen and electron microscopy of the diseased and healthy fruiting bodies of *L. edodes*.

Materials and Methods

A strain of *P. tolaasii* (TMI 32282, Tottori Mycological Institute), which had been isolated from a diseased fruiting body of *L. edodes* on a bedlog of *Quercus serrata* Thunb. in Mie prefecture, was used throughout this study. Eight other strains of *P. tolaasii* were examined for comparison of bacteriological characteristics: OL1 of Tokyo University of Agriculture (TUA), isolated from *L. edodes*; TMI 32228 and TUA-MT 93001, both isolated from *A. brunnescens*; TMI 32230 and TUA 814, both isolated from *P. ostreatus*; and TUA-PT 9401, 9402, 9403, all from *F. velutipes*. All of these cultures were maintained on Pseudomonas agar F (PAF; Difco, Detroit, Mich.). Bacteriological characteristics of these strains were determined using the methods described by Pelezar (1957), Lelliott et al. (1966), Nishiyama (1978) and Schaad (1988).

Both white-line and rapid pitting tests were carried out by the methods of Wong and Preece (1979). In the white-line test, two strains of *Pseudomonas* sp. (TUA-WLRO-N 9401, TMI 32233) were employed as testers. For the rapid pitting test, fresh fruiting bodies of *L. edodes* (TMI 800) were harvested and tissue blocks (ca. 0.5 × 1.0 cm) were cut from their caps after removing the outer tissues. One loopful of bacterial suspension (10^8–10^9 cells/ml) prepared from a young culture of *P. tolaasii* (TMI 32282) was placed onto the cut surface of the tissue block. Sterile distilled water was applied to controls instead of bacterial suspension. The inoculated tissue blocks were placed on sterilized glass microscope slides in a glass Petri dish (four inoculated and two control tissue blocks per dish) lined with a moistened filter paper. They were then incubated at 5, 10, 15, 20 or 25°C in the dark for 24 to 48 h and examined for the occurrence of browning and sunken lesions. Both white-line and rapid pitting tests were repeated three times, using *P. tolaasii* cultures that had been stored in liquid nitrogen until use.

Diseased fruiting bodies, collected from *Q. serrata* bedlogs at Ohmiya-cho, Mie prefecture in December,

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Table 1. Characteristics of *Pseudomonas tolaasii* (TMI 32282).

Shape of cells	rod	lecithinase	+
Endospores	none	oxidase	+
Respiration	aerobic	tyrosinase	+
Growth at 41°C	−	Gelatin liquefaction	+
Gram stain	−	Hydrolysis of Tween-80	+
Production of		Oxidation of gluconic acid	+
fluorescent pigment	+	Nitrate reduction	+
levan	−	Utilization of	
Induction of		glucose	+
potato rot	−	sucrose	−
tobacco hypersensitive reaction	−	sodium tartrate	−
Enzymatic activity of		sodium benzoate	−
arginine dihydrolase	+	sodium hippurate	−
catalase	+	White-line formation	+

+: positive, −: negative.

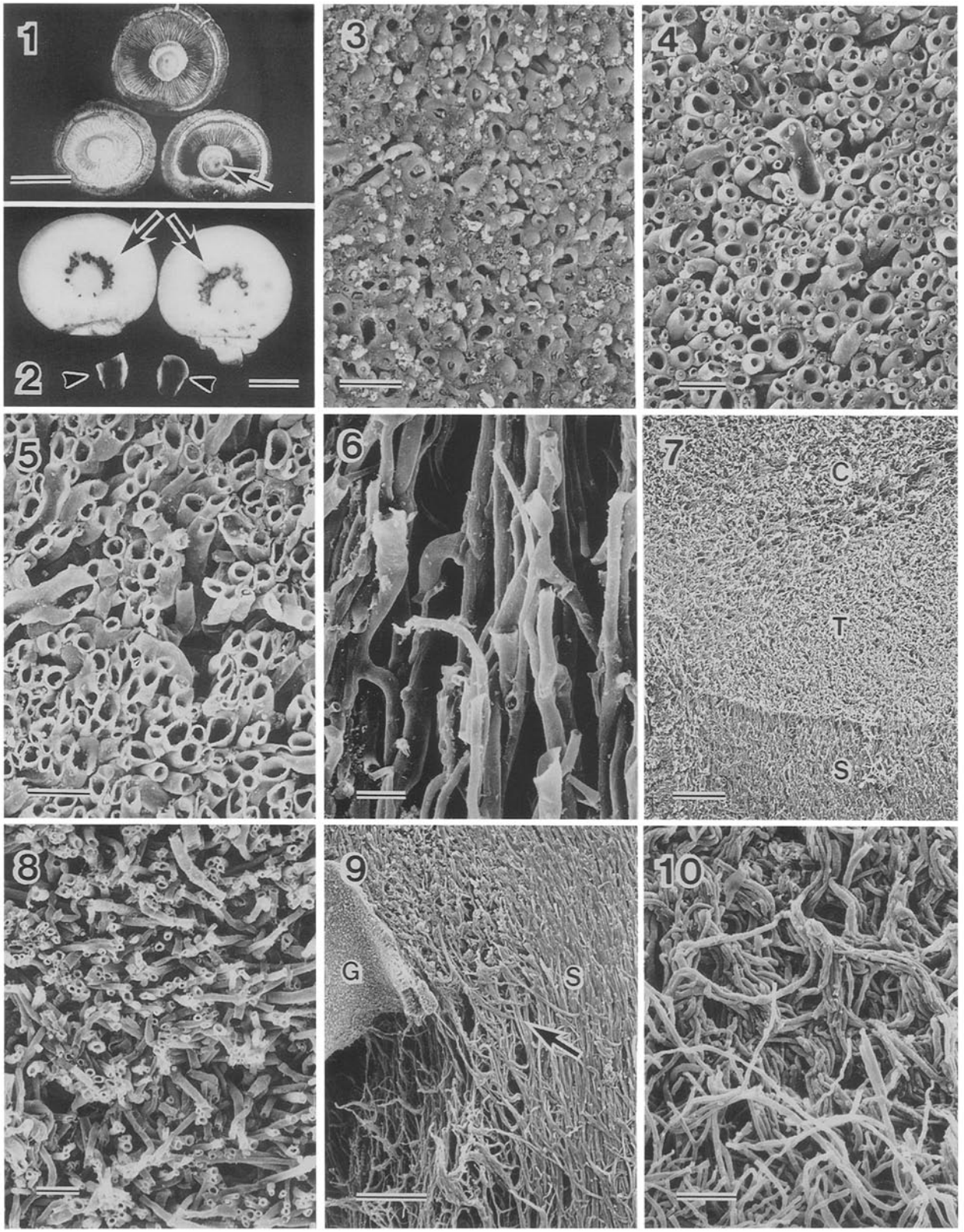
1993, were used for electron microscopy. Some of them were covered with snow when harvested. Healthy fruiting bodies of various sizes were also examined for comparison. Interaction studies were conducted with *L. edodes* grown in wood or inner bark pieces of *Q. serrata*, incubated in sawdust flask culture (Tsuneda et al., 1991). Pieces of inner bark (ca. 8×8×8 mm) were prepared by cutting outer sapwood bearing inner bark from a *Q. serrata* log and were air-dried under laboratory conditions until use. Cylinders of *Q. serrata* sapwood, 8 mm in diam and approximately 8 mm long, were used to make wood pieces: half of them were split along the radial plane and the other half were split tangentially along a line of early-wood vessels. All of these inner bark and wood pieces were soaked overnight in distilled water, drained and then autoclaved 30 min at 121°C before being added to the sawdust flask culture of *L. edodes*. After incubation at 25°C for one month, the inner bark and wood pieces bearing *L. edodes* mycelium were inoculated with *P. tolaasii* and incubated at 25°C for 7 days. Inoculation and culture methods were the same as those used in the rapid pitting tests. Materials for scanning (SEM) and transmission (TEM) electron microscopy were prepared by the methods described previously (Tsuneda and Thorn, 1995).

Results and Discussion

Bacteriological characteristics Cells of TMI 32282 are rod-shaped and contain no endospores. They are negative in Gram staining and aerobic. Colonies on PAF medium are milky white with irregular edges, and produce fluorescent, diffusible pigment(s) into agar that become greenish yellow to reddish brown in old cultures. Inoculation of this bacterium causes blackening of potato tubers but not their rot, and it does not induce hypersensitive necrosis of tobacco leaves. Some other important bacteriological characteristics of this strain are listed in Table 1. All of these characteristics agreed well with those of other strains of *P. tolaasii* examined in this study for comparison. In addition, results of the white-line test were positive on PAF medium, although this strain tended to lose its ability to induce white lines after several transfers onto PAF medium: this aspect was confirmed using cultures that had been stored in liquid nitrogen. Analyses of rDNA of TMI 32282 also indicated that it is a strain of *P. tolaasii* (Thorn, G. and Tsuneda, A., unpublished data).

Development of symptoms on inoculated and naturally infected fruiting bodies Inoculation with *P. tolaasii* caused browning, pitting (Wong and Preece, 1979) and sunken lesions of the cut surfaces of cap and stalk tissue blocks of *L. edodes* (Table 2). Tissue browning was not recognized or negligible at 5°C within 48 h after inoculation, but it developed at more or less equal intensity above 10°C. In general, pitting and sunken lesions were more pronounced on cap than stalk tissues, and these symptoms were apparent even at 5°C. These results explain the fact that this disease occurred during winter in the field where some of the infected fruiting bod-

Figs. 1–10. Fruiting bodies of *Lentinula edodes*, healthy or infected by *Pseudomonas tolaasii*. 1. Browning of gills and stalk tissues. Necrosis is most severe in the central part of stalks (arrow). A healthy specimen is shown on the left. Bar=3 cm. 2. Cut surfaces of infected caps and stalks. Note necrotic cavities developed in the periphery of the area of attachment to the stalk (arrows). Inside the stalks are darkly browned (arrowheads). Bar=2 cm. 3–10. Internal tissue organization of healthy fruiting bodies. 3–5. Transverse sections of a stalk, 2 mm above its extreme bottom; about 200 μm (3), 400 μm (4) and 5 mm (5) inside from the outer edge. Bars=20 μm. 6. Longitudinal and loose hyphal arrangement at the central portion of a stalk. Bar=20 μm. 7. Transition layer (T) present between the cap (C) and stalk (S) tissues. Bar=300 μm. 8. Tightly packed and randomly arranged hyphae in the transition layer. Bar=20 μm. 9. Channel-like area (arrow) between a stalk (S) and a gill (G). Bar=200 μm. 10. Spongy cap tissue. Bar=50 μm.


1
2
3
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7
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G
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Table 2. Browning and lysis of fruiting body tissues of *Lentinula edodes*[1] caused by *Pseudomonas tolaasii*[2] at different temperatures.

		5°C		10°C		15°C		20°C		25°C	
		cap	stalk	cap	stalk	cap	stalk	cap	stalk	cap	stalk
	Symptom[4]										
24 h[3]	Br	−	−	+	+~++	+~++	+	++	++	++	++
	Pt	+	±	+	±	+	+	++~+++	+	++	+
48 h	Br	−	±	+	+++	+~++	++	++	++	++	++
	Pt	++	+	+~++	+	+~++	+	+++	++	++	++

[1] TMI 800. [2] TMI 32282. [3] Incubation time (h).

[4] Br: browning of the inoculated tissue, Pt: pitting and sunken lesions of the inoculated tissue. Development of the symptom: −; nil, ±; nil or slight on some samples, +; slight, ++; distinct, +++; severe. No symptom developed on control tissues except that browning of stalk tissues occurred at 25°C.

ies were covered with snow when harvested. Regardless of mushroom species, browning and pitting are common symptoms induced by *P. tolaasii* (Wong and Preece, 1979; Rainey et al., 1991; Suyama and Fujii, 1993; Tsuneda and Thorn, 1994). This bacterium species is known to produce a lipodepsipeptide toxin, named tolaasin (Nutkins et al., 1991) that induces both of these symptoms in *A. brunnescens* (Rainey et al., 1991).

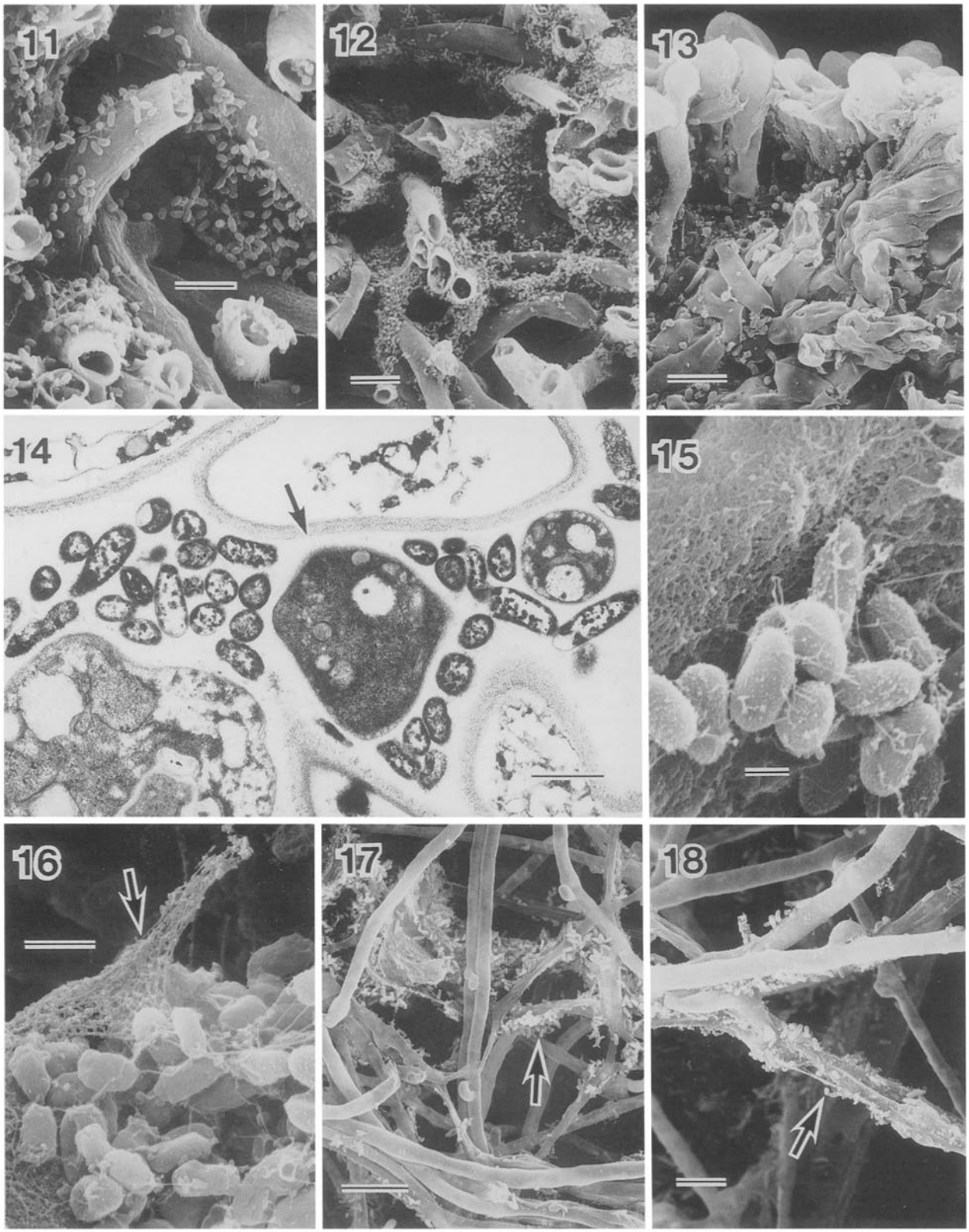
Control and naturally infected fruiting bodies are shown in Figs. 1 and 2. In general, the infected fruiting bodies are readily distinguishable from healthy ones by browning of gills and stems, although the intensity of browning varies with fruiting bodies. At the base of infected stems, the degree of tissue necrosis is usually strongest at the central part (Fig. 1), but above the basal region almost the entire width of the infected stalk becomes evenly necrotic up to the point of attachment to the cap (Fig. 2, arrowheads). Necrosis does not usually develop at the attachment area, but dark brown necrotic cavities occur in the cap tissue just above the periphery of the attachment area (Fig. 2, arrows). Then necrosis spreads inside the cap. To explain the mechanism of this mode of symptom development, the internal structure of *L. edodes* fruiting bodies was examined.

Electron microscopy of healthy and naturally infected fruiting bodies Stalks of *L. edodes* fruiting bodies are narrowed and embedded in the bark of bedlogs at their bases. The basal end part is composed of a compacted mass of randomly oriented hyphae. Hyphae gradually become arranged longitudinally above this point. Shown in Figs. 3–5 are portions of a transversely cut surface of a mid-sized stalk, 2 mm above the extreme end. At the peripheral region, hyphae are densely packed and arranged more or less longitudinally. They are mostly thick-walled, variable in diam and cemented to each other with amorphous matrix materials (Fig. 3). The amount of the matrix materials becomes much less at 300 to 400 μm inside from the outer edge (Fig. 4). Occurrence of thick-walled fine hyphae also become progressively less frequent toward the center (Figs. 3–5). Apparently, these structural features explain the fact that the central portion is most susceptible to bacterial attack and proliferation, and therefore often show the strongest signs of necrosis (Fig. 1).

Above the basal region, component hyphae are relatively sparse, thin-walled and arranged longitudinally (Fig. 6) up to the transition layer that is present between stalk and cap tissues. The transition layer is mostly from 1 to 2 mm thick and consists of tightly interwoven hyphae (Figs. 7, 8). This layer blocks bacterial invasion toward the cap tissue. In contrast, loosely arranged hyphae occur in the attachment region between the periphery of the transition layer and edges of gills, giving this area a channel-like appearance under SEM (Fig. 9; arrow). Apparently, this circumferential boundary area provides an important pathway for bacteria to the spongy cap tissue (Fig. 10), and therefore necrotic cavities develop along this boundary area (Fig. 2).

Bacterial multiplication occurs intercellulary regardless of the portion of infected fruiting bodies (Figs. 11–14). Examinations of many thin sections of infected fruiting body tissues revealed no evidence of intracellular occurrence of the bacterium. Cells of *L. edodes* surrounded by bacterial cells become electron-dense first and soon degenerate (Fig. 14). These results differ strikingly from those reported previously for an unidentified rod-shaped bacterium that is also pathogenic to *L. edodes* fruiting bodies (Nakai et al., 1982). Occurrence of this bacterium was strictly intracellular and bacterial cells were occasionally found even within hyphal cells containing apparently intact nuclei, mitochondria and ground cytoplasm. Similar intracellular occurrence of *Pseudomonas* sp. was demonstrated in mummy-diseased fruiting bodies of *A. brunnescens* (van Zaayen and Waterreus, 1974). Whether the multiplication of *P. fluorescens* that was reported to cause browning and rot of *L. edodes* fruiting bodies (Komatsu and Goto, 1974) was intercellular or intracellular is not known. Bacterial diseases of mushrooms may provide an excellent system for the study of mechanisms that determine the modes of microbial multiplication within host tissues. It requires less time and space to create pathogenic conditions, and uniformity of materials is superior to that of most other systems, such as the higher plant-fungal system.

Degradation of host hyphal cell walls by *P. tolaasii* occurs (Figs. 15, 16) in heavily infected areas of *L. edodes* fruiting bodies. In the initial stages of degradation, amorphous coating materials tend to be removed preferential-



Figs. 11–18. Electron microscopy of infected fruiting bodies and lysed hyphae of *Lentinula edodes* by *Pseudomonas tolaasii*. 11–14. Intercellular multiplication of bacterial cells in the stalk (11), cap (12, 14) and hymenium (13). The arrow in Fig. 14 points to an affected cell of *L. edodes* that has become electron-dense. Bars = 5 μm (11, 13); 10 μm (12); 1 μm (14). 15, 16. Early and late stages of hyphal cell wall lysis observed in an infected cap. The arrow in Fig. 16 indicates microfibrillar bundles of the host hypha. Bars = 0.5 μm (15); 1 μm (16). 17, 18. Lysed host hyphae (arrows) in inner bark (17) and outer sapwood (18). Bars = 10 μm (17); 5 μm (18).

ly from the hyphal cell walls, and thus bundles of microfibrils are exposed (Fig. 15). These microfibrillar bundles are then lysed by bacteria (Fig. 16). This mode of cell wall degradation, however, is milder than that reported previously for a different strain of *P. tolaasii* (TMI 32237) that was isolated from a diseased fruiting body of *P. ostreatus* (Tsuneda and Thorn, 1994): this strain lysed cap tissues of *L. edodes* more rapidly and degraded all the wall components nearly simultaneously. On the other hand, a strain from a diseased fruiting body of *F. velutipes* was weak or negligible in its lytic activity against *L. edodes* hyphae (Tsuneda and Thorn, 1994). These results suggest that strength of pathogenicity and host range of *P. tolaasii* strains are determined, at least in part, by their productivity and (or) the kind of enzymes that are involved in the lysis of host cell walls. The preferential and simultaneous types of cell wall degradation also occur in some mycoparasitic as well as wood decay processes (Otjen and Blanchette, 1986; Tsuneda and Thorn, 1995). Recently, an endo-β-1,6-glucanase from a mycoparasitic strain of *Trichoderma harzianum* Rifai was purified and shown to contribute to an effective degradation of filamentous fungal cell walls when combined with other *T. harzianum* cell wall degrading enzymes such as β-1,3-glucanases and chitinases (De la Cruz et al., 1995).

Interaction on inner bark and sapwood of *Quercus serrata* According to Suyama and Fujii (1993), artificial inoculation of *L. edodes* fruiting bodies with *P. tolaasii* caused browning of the fruiting bodies and also the mycelium within wood. In the present study, we applied a bacterial solution of *P. tolaasii* to the healthy mycelium of *L. edodes*, which had been previously grown on either inner bark or sapwood pieces, and confirmed that the bacterium is able to attack vegetative hyphae as well. Heavily infected hyphae are lysed by bacteria in the same fashion as fruiting body tissues (Figs. 17, 18; arrows). This indicates that *P. tolaasii* is capable of spreading along the mycelium in the inner bark and sapwood regions and causes infections of healthy fruiting bodies, including primordia, on the bedlog.

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Literature cited

De la Cruz, J., Pintor-Toro, J. A., Benitez, T. and Llobell, A. 1995. Purification and characterization of an endo-β-1,6-glucanase from *Trichoderma harzianum* that is related to its mycoparasitism. J. Bacteriol. **177**: 1864–1871.

Fermor, T. R. 1987. Bacterial diseases of edible mushrooms and their control. In: "Cultivating edible fungi," (ed. by Wuest, P. J., Royse, D. J. and Beelman, R. B.), pp. 361–370. Elsevier, Amsterdam.

Fletcher, J. T., White, P. F. and Gaze, R. H. 1989. "Mushrooms: pest and disease control, 2nd ed.," Intercept, Ponteland. 174p.

Komatsu, M. and Goto, M. 1974. Bacterial disease of cultivated shiitake-mushroom, *Lentinus edodes* (Berk.) Sing. in Japan. Rept. Tottori Mycol. Inst. **11**: 69–82. (In Japanese.)

Lelliott, R. A., Billing, E. and Hayward, A. C. 1966. A determinative scheme for the fluorescent plant pathogenic pseudomonads. J. Appl. Bacteriol. **29**: 470–489.

Nakai, Y., Ushiyama, R. and Komatsu, M. 1982. Presence of a rod-shaped bacterium in *Lentinus edodes* fruit-bodies with a browning symptom. Rept. Tottori Mycol. Inst. **20**: 47–53.

Nishiyama, K. 1978. A proposal of a simple identification method for plant pathogenic bacteria. Plant Protection (Shokubutu Bôeki) **32**: 283–288. (In Japanese.)

Nutkins, J. C., Mortishire-Smith, R. J., Packman, L. C., Brodey, C. L., Rainey, P. B., Johnstone, P. and Williams, D. H. 1991. Structure determination of tolaasin, an extracellular lipodepsipeptide produced by the mushroom pathogen *Pseudomonas tolaasii* Paine. J. Amer. Chem. Soc. **113**: 2621–2627.

Otjen, L. and Blanchette, R. A. 1986. A discussion of microstructural changes in wood during decomposition by white rot basidiomycetes. Can. J. Bot. **64**: 905–911.

Pelezar, M. J. J. (ed.). 1957. "Manual of microbiological methods," Society of American Bacteriologists Committee on Bacteriological Technic. McGraw-Hill Book, New York. 315 p.

Rainey, P. B., Brodey, C. L. and Johnstone, K. 1991. Biological properties and spectrum of activity of tolaasin, a lipodepsipeptide toxin produced by the mushroom pathogen *Pseudomonas tolaasii*. Phys. Molec. Pl. Pathol. **39**: 57–70.

Schaad, N. W. 1988. "Laboratory guide for identification of plant pathogenic bacteria, 2nd ed.," APS Press, St. Paul. 158 p.

Suyama, K. and Fujii, H. 1993. Bacterial disease occurred on cultivated mushroom in Japan. J. Agri. Sci. Tokyo Nogyo Daigaku **38**: 35–50. (In Japanese.)

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

Quercus serrata sapwood by *Lentinus edodes* and its inhibition by an antagonistic hyphomycete, *Leptodontidium elatius*. Can. J. Bot. **25**: 36–48.

Tsuneda, A. and Thorn, R. G. 1995. Interactions of wood decay fungi with other microorganisms; with emphasis on the degradation of cell walls. Can. J. Bot. **73**: In Press.

Tsuneda, A. and Thorn, R. G. 1994. Interactions between *Lentinula edodes* and pseudomonads. Can. J. Microbiol. **40**: 937–943.

Tsuneda, A., Maekawa, N., Ohira, I. and Furukawa, I. 1991. Incipient decay of van Zaayen, A. and Waterreus, H. A. J. I. 1974. Intracellular occurrence of bacteria in mummy-diseased mushrooms. Phytopathology **64**: 1474–1475.

Wong, W. C. and Preece, T. F. 1979. Identification of *Pseudomonas tolaasii*: the white line in agar and mushroom tissue block rapid pitting. J. Appl. Bacteriol. **47**: 401–407.