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Occurrence of Internal Stipe Necrosis of Cultivated Mushrooms (*Agaricus bisporus*) Caused by *Ewingella americana* in Korea

Chan-Jung Lee[1]*, Chang-Sung Jhune[1], Jong-Chun Cheong[1], Hyung-Sik Yun[1] and Weon-Dae Cho[2]

[1]*Mushroom Research Division, National Institute of Horticultural & Herbal Science, RDA, Suwon 441-707, Korea*
[2]*National Academy of Agricultural Science, RDA, Suwon 441-707, Korea*

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The internal stipe necrosis of cultivated mushrooms (*Agaricus bisporus*) is caused by the bacterium *Ewingella americana*, a species of the Enterobacteriaceae. Recently, *Ewingella americana* was isolated from cultivated white button mushrooms in Korea evidencing symptoms of internal stipe browning. Its symptoms are visible only at harvest, and appear as a variable browning reaction in the center of the stipes. From these lesions, we isolated one bacterial strain (designated CH4). Inoculation of the bacterial isolate into mushroom sporocarps yielded the characteristic browning symptoms that were distinguishable from those of the bacterial soft rot that is well known to mushroom growers. The results of Gram stain, flagellal staining, and biochemical tests identified these isolates as *E. americana*. This was verified by pathogenicity, physiological and biochemical characteristics, and the results of an analysis of the 16S rRNA gene sequences and the fatty acids profile. This is the first report of the isolation of *E. americana* from cultivated white button mushrooms in Korea.

KEYWORDS : *Agaricus bisporus*, *Ewingella americana*, Internal stipe necrosis, Mushroom, 16S rRNA gene

The button mushroom, *Agaricus bisporus,* is one of the most popular mushrooms in cultivation worldwide, and is one of the major economically important crops cultivated in Korea. The production areas have decreased somewhat, by approximately 196 ha in 2004 to 176 ha in 2005. Internal stipe necrosis has been previously recognized as a major problem within the mushroom industry (Inglis *et al*., 1996). *Ewingella americana*, a bacterium belonging to the Enterobacteriaceae, has been previously associated with a browning disorder of the stipe of the mushroom *Agaricus bisporus*, which is called internal stipe necrosis (Inglis, 1995; Inglis *et al*., 1996; Roy Chowdhury *et al*., 2007). The symptoms of internal stipe necrosis appear as a variable browning reaction in the center of the mushroom stipe, and some specimens may also evidence a slight browning of internal cap tissue which abuts the stipe core. The disease caused by this potentially serious mushroom pathogen was initially described as internal stipe necrosis of mushrooms in the United Kingdom (Inglis and Peberdy, 1996; Inglis *et al*., 1996) and this pathogen was later detected on mushrooms in New Zealand (Braithwaite *et al*., 2005). However, this bacterial disease has not yet been reported in Korea.

Recently, *E. americana* was isolated from cultivated button mushrooms in Korea which exhibited symptoms of internal stipe browning. Its pathogenicity was confirmed. Herein, we report the occurrence of internal stipe necrosis on the button mushroom, *Agaricus bisporus*, in Korea as a new finding, and present our findings regarding the phenotypic, biological, and pathological characteristics of the isolate.

Disease occurrence and symptoms. A bacterium responsible for dark brown symptoms has been isolated consistently from mushrooms with stipe necrosis. The primary symptom is a mild browning reaction in the centers of the stipes. As the mushrooms mature, affected tissues may collapse, after which the collapsed parts will change color, turning a very dark brown shade. Infected mushrooms often appear externally sound, but when cut longitudinally, young sporocarps are revealed, which have a dark brown color. The symptoms are visible only at harvest and appear as a variable browning reaction in the centers of the stipes, and may be accompanied by a limited collapse of the internal tissues--when the stipe is removed, a characteristic brown peg of dead cork-like tissue remains attached to the pileus (Fig. 1A, B).

Bacterial isolation and pathogenicity. Button mushrooms evidencing internal stipe necrosis symptoms were obtained in 2007~2008 from mushroom cultivation facilities in Korea. For the isolation of the causal pathogen, approximately 1 g of infected mushroom stipe was crushed in sterilized 1.5 *ml* Eppendorf tubes containing 1 *ml* of distilled water. The crushed mushroom suspension was diluted in series, plated onto R2A agar and incubated at 28°C. Bacterial colony was then streaked separately onto R2A agar or King's B and maintained at −70°C in Luria-

*Corresponding author <E-mail : lchanj@rda.go.kr>

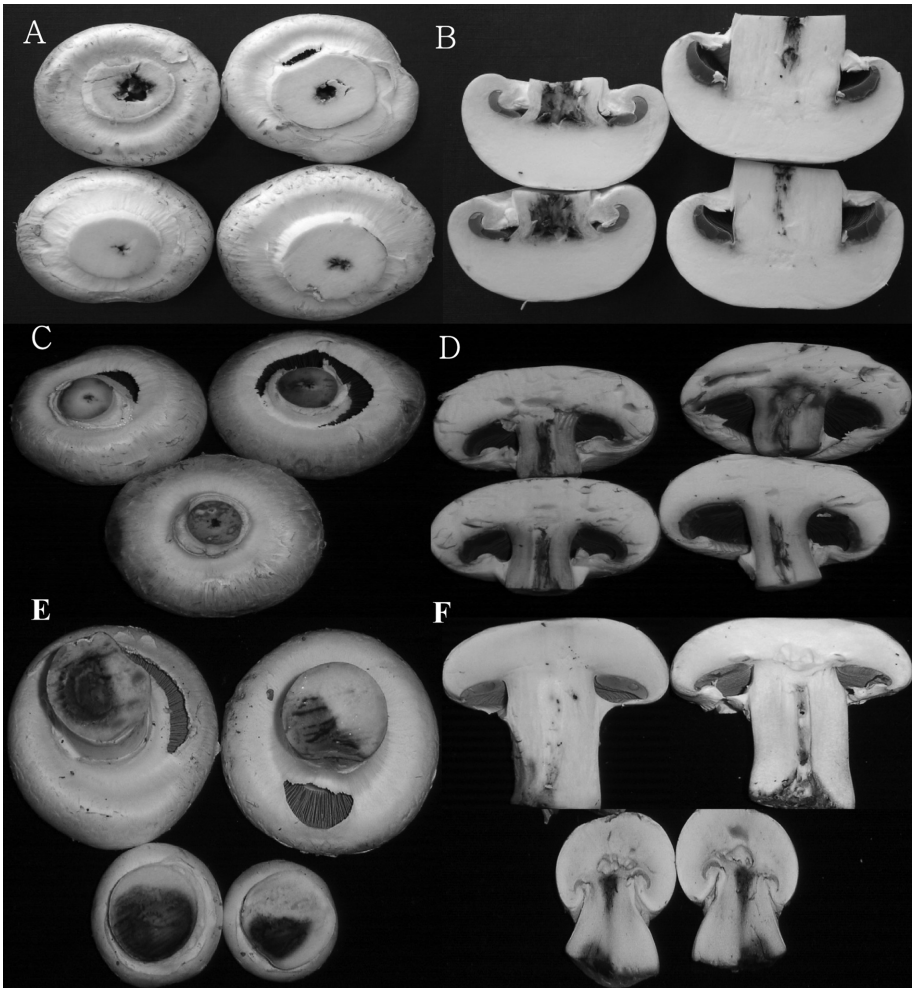

Fig. 1. Symptoms of internal stipe necrosis of cultivated mushrooms. A and B, typical symptoms of internal stipe necrosis on naturally infected button mushrooms; C and D, tissue necrosis on mushroom stipes inoculated with *Ewingella americana* into the stipes of button mushrooms at the time of harvest; E and F, tissue necrosis on mushroom stipes injected with *E. americana* into the stipe bases of young mushrooms at the 'pin' stage.

Bertani medium (LB) (Difco Laboratories, Detroit, MI, USA) containing 15% (v/v) glycerol for further use.

We selected one isolate (CH4) from several colonies with the same colony morphology as a type culture for further studies. Bacterial isolates grown for 1~2 days on R2A agar were collected in sterile distilled water, and the concentrations were adjusted to 5×10^8 cfu/*ml* as an inoculum for the pathogenicity tests. The test of pathogenicity to the host was conducted via the injection of a suspension bacterium directly into the stipe (Inglis *et al.*, 1996), using healthy cultivated mushrooms. The first injection trial involved injection with a sterilized needle into the stipe tissue at the time that the button mushrooms were harvested. After inoculation, the mushrooms were incubated for 3 days at ambient temperature (23~25°C) and examined for symptoms (stipe brown lesions). A second trial involved injection with a sterilized needle into the stipe bases of young mushrooms at the 'pin' stage; and these mushrooms were marked on the caps for later identification. The control treatments consisted of a batch of mushrooms injected with sterile water alone.

The CH4 strain was inoculated in the stipes via the injection of a bacterial suspension, which caused typical browning disorders and necrosis along the length of the inoculation point (Fig. 1. C, D, E, F). The central core of stipe tissue around the inoculation points initially became a mild brown color and then collapsed-after which the collapsed parts eventually developed a very dark brown color and were 'typically' destroyed. The water-inoculated controls evidenced no such browning symptoms. Following the successful reinfections, it became clear that the bacterium was pathogenic (Fig. 1D, F). Koch's Postulates were satisfied and the pathogenicity of the isolates was established. The browning and internal stipe necrosis observed in *A. bisporus* corresponds with that previously described by Inglis *et al.* (1996) and Roy Chowdhury *et al.* (2007). These isolates were deposited in KACC (strain CH4; KACC13095). The type species of *Ewingella amer-*

icana KCTC 12690, used for comparison, was obtained from the Korean Collection of Type Cultures.

Identification of the bacterial isolate. Colony morphology was assessed on King's B agar or Biolog Universal Growth agar (Biolog Inc., CA) as previously described by Roy Chowdhury *et al.* (2007). The morphology of the bacterial cells was also evaluated using electron microscopy after staining with 2% phosphotunstic acid (PTA, pH 7.0). For the identification of the bacterium the following cultivation techniques and biochemical tests were employed: Gram staining, presence of oxidase, growth in KCN, starch hydrolysis and gelatinase activity (Cowan and Steel, 1965), nitrate reduction (Lanyi, 1987), indole production, lipase and DNAase activity, and acid production from different sugars (Dowson, 1957). Utilization of D-glucose, amylose, lactose, mannitol, galactose, D-fructose, D-mannose, L-tyrosin, ammonium citrate, ribose, N-acethyl glucosamine, arbutin, esculin, salicin, cellobiose, maltose, lactose, trehalose, β-gentiobiose, D-arabitol, 5-ceto-guconate, malonate, L-arabinose, inulin, urea, ammonium chloride, D-sorbitol, D-raffinose, tartrate, arginine, betain, acetate, erythritol, rhamnose, sucrose, butylamine, meso-tartrate, n-amylamine and glycogen were tested on basal medium, as described elsewhere (Stanier *et al.*, 1966). Additional enzyme activities and biochemical properties were assessed using the API 20E, API 20NE, rapid ID 32A, API 50CH and API ZYM systems (bioMçrieux); the utilization of various substrates was determined via inoculation of the API 50CH strip with cells suspended in AUX medium (bioMērieux). The rapid ID 32A and API ZYM tests were read after 4 h of incubation at 37°C, and the others were read after at least 48 h at 30°C.

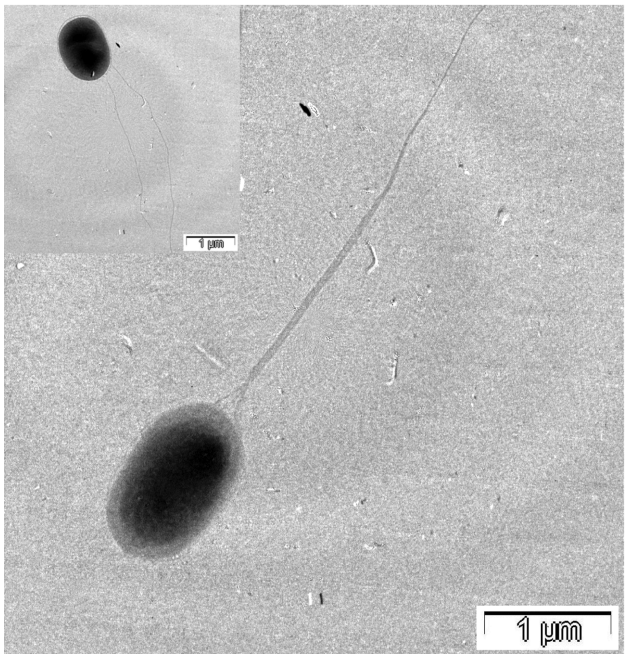

Fig. 2. Transmission electron micrographs of *Ewingella americana* showing a rod-shaped bacterium with peritrichous flagella (scale bar = 1.0 μm).

The colonies on King's B agar were round, brown with small papillae, non-fluorescent and 1.5~2.5 mm in diameter after 2 days of incubation at 30°C. Bacterial cells observed by electron microscopy were rod-shaped, with rounded ends, peritrichous flagella, and an average size of 1.3~1.6 × 0.6~0.8 μm (Fig. 2).

The tested CH4 isolate was a Gram-negative rod, Voges-Proskauer and ONPG positive, DNAase, urease, oxidase, arginine dehydrolase, gelatin hydrolysis, and ornithine decarboxylase negative. It reduced nitrates to nitrites, grew in chitin (CM-RBV) and 5% NaCl, grew very slowly at 5°C but did not grow at 40°C or in KCN. It induced hydrolysis with Tween 80 but did not fluoresce on KB medium. Indole and hydrogen sulphide were not produced. It utilized D-glucose, D-galactose, glycerol, amylose, lactose, mannitol, galactose, D-fructose, D-mannose, D-mannitol, ammonium citrate, ribose, trisodium citrate, N-acethyl glucosamine, arbutin, esculin, salicin, D-cellobiose, D-maltose, D-lactose, D-ribose, D-trehalose, gentiobiose, β-gentiobiose, D-arabitol, n-amylamine, butylamine, meso-tartrate, 2-ketoguconate and 5-ketoguconate but not malonate, D-arabinose, L-arabinose, inulin, urea, ammonium chloride, D-sorbitol, D-raffinose, D-tartrate, arginine, betain, acetate, meso-erythritol, L-rhamnose, sucrose, glycogen, xylitol, and D-tryptophan.

It produced acid from the sugars D-glucose, D-galactose, D-fructose, D-maltose, D-mannose, ribose and mannitol, but failed to generate acid from amylose, D-xyrose, inulin, D-lactose, L-tyrosine, D-sorbitol, L-rhamnose, D-raffinose, L-arabinose, melibiose, raffinose, D-sorbitol, or sucrose. In assays with the API ZYM and rapid ID 32A systems, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, alkaline phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, N-acetyl-β-glucosaminidase, β-galactosidase-6phosphate, arginine arylamidase, proline arylamidase, leucyl glycine arylamidase, phenylalanine arylamidase, pyroglutamic acid arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase, histidine arylamidase, serine arylamidase were present, but valine arylamidase, lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, β-galactosidase, α-galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase, α-fucosidase, β-arabinosidase and glutamyl glutamic acid arylamidase were not detected. The antibiotic sensitivity test was conducted via the disk diffusion method (Roy Chowdhury *et al.*, 2007). The CH4 isolate evidenced susceptibilities to ampicillin (100 mg/*ml*), nalidixic acid (15 mg/*ml*), kanamycin (50 mg/*ml*), streptomycin (50 mg/*ml*), tetracycline (15 mg/*ml*), gentamycin (30 mg/*ml*), chloramphenicol (30 mg/*ml*), rifampicin

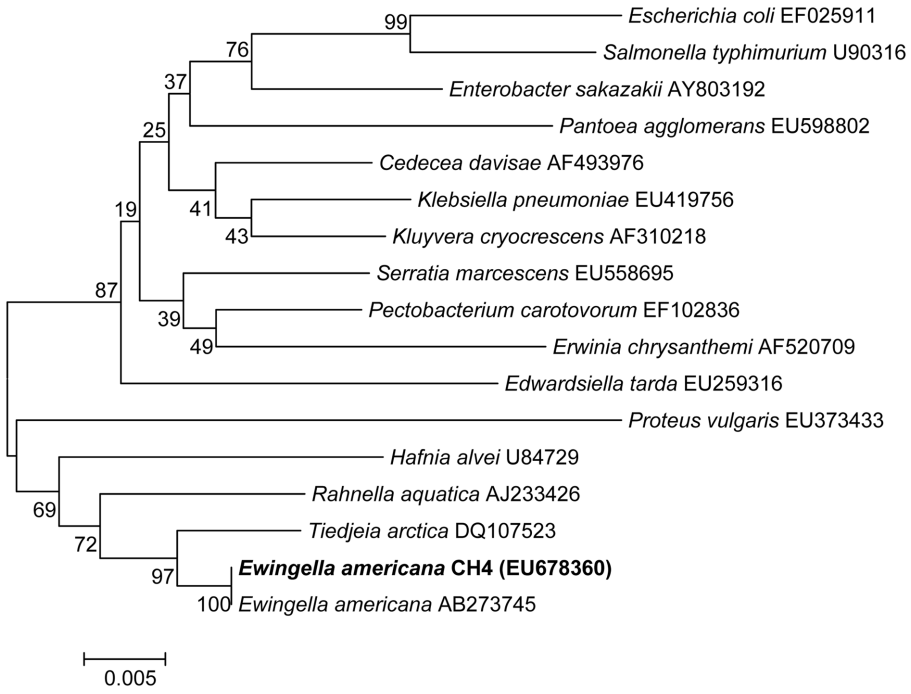

Fig. 3. Phylogenetic distance tree constructed by the neighbor-joining method, comparing the 16S rDNA sequences of the isolate (CH4) from this study and members of the Enterobactereciae group from GenBank. Accession numbers are provided in parentheses. The bar represents a phylogenetic distance of 0.5%.

(50 mg/*ml*), neomycin (15 mg/*ml*), but was resistant to cephalothin (50 mg/*ml*). For fatty acid methyl ester analysis, cells were harvested from trypticase soy agar (TSA; Difco) plates after 2 days of incubation at 30°C. The fatty acids were extracted and fatty acid methyl esters were prepared in accordance with the standard protocols of the MIDI Hewlett-Packard Microbial Identification System (Sasser, 1990). FAME analysis on cellular fatty acid compositions of the CH4 strain identified the isolate as *E. americana* with a 0.75 similarity index in the library database [the Microbial Identification System Library for aerobes (ver. 3.90)] (data not shown). The major fatty acid components of the examined CH4 strain were as follows: 16:0 (31.9%), 17:0cyclo (22.2%), 18:1w7c (11.8%) and sum in feature 2 (9.6%, comprising 14:3 OH/16:1 iso I). Sequencing and assembly of the 16S rRNA gene were conducted as previously described (Lane, 1991). The resultant 16S rRNA gene sequence (1392nt) of the CH4 strain was compared with the 16S rRNA gene sequences available from GenBank, using the BLAST program to determine an approximate phylogenetic affiliation, after which the gene sequence was multiply aligned with those of closely related species using the MEGALIGN program (DNASTAR). Phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei, 1987) with the MEGA3.1 program (Kumar *et al*., 2004); bootstrap percentages were based on 1000 replications (Felsenstein, 1985). This strain evidenced 99% 16S rRNA sequence similarity with *E. americana,* according to our BLAST search for homologous sequences (GenBank accession No. EU678360). Fig. 2 shows the inferred phylogenetic relationships derived from a neighbor-joining analysis among the 16S rRNA gene sequences of CH4 isolates from this survey with 17 validly described Enterobacteriaceae species. The CH4 isolates from this study were most closely related to *E. americana* (Fig. 3).

The bacterial pathogen isolated from the internal stipe necrosis of white button mushrooms was identified as *E. americana*. In the pathogenicity test, this bacterium induced typical browning disorders and necrosis along the length of the inoculation point (Fig. 1. C, D). However, this disease was never observed to have penetrated the upper pileal surface. The central core of stipe tissue around the inoculation points initially became a mild brown color and collapsed, after which the collapsed parts assumed a very dark brown color, and were 'typically' destroyed. Inglish and Peberdy (1996) reported the first non-animal isolation of *E. americana* in cultivated mushrooms, *A. bisporus*, in the United Kingdom, where *E. americana* was reported to be the pathogenic agent of internal stipe necrosis (Inglis *et al*., 1996) *E. americana* is the sole member of a relatively recently recognized bacterial genus, *Ewingella* (Eeterobacteriaceae), as proposed by Grimont *et al*. (1983), and previously referred to as enteric group 40. As we follow the current bacterial nomenclature system, this is the first report in Korea showing that

E. americana is the causal pathogen of internal stipe necrosis of the button mushroom, *Agaricus bisporus*.

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