Evidence for the association of the enteric bacterium *Ewingella americana* with internal stipe necrosis of *Agaricus bisporus*

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Internal stipe necrosis of *Agaricus bisporus* is recognized as an emerging and potentially serious disease in the UK mushroom industry. Symptoms are visible only on harvest and appear as a variable browning reaction in the centre of stipes, which may be accompanied by limited collapse of the internal tissues. The hypothesis that this problem is of bacterial origin was investigated, initially by an extensive bacteriological examination of affected mushrooms. The enteric bacterium *Ewingella americana* was isolated from at least 93% of symptomatic mushrooms. Various strains of *Pseudomonas fluorescens* are also usually present in diseased mushrooms, but no single *P. fluorescens* biovar was consistently associated with the disease. Typical symptoms were reproduced following infection trials with isolates of *E. americana* derived from diseased mushrooms. In addition, strains recovered from diseased mushrooms following such trials were shown by RFLP studies to be identical to those applied, thereby confirming Koch's postulates for these strains. The possible contribution of other bacterial species to symptom development is discussed.

Keywords: *Agaricus bisporus*, *Ewingella americana*, Koch's postulates, internal stipe necrosis, mushroom disease

INTRODUCTION

The button mushroom, *Agaricus bisporus* is the most popular mushroom in cultivation world-wide, and is the most important protected crop in the UK, with a wholesale value probably exceeding £250 million. Recently, internal stipe necrosis (ISN) has been recognized as an emerging problem within the mushroom industry. Outbreaks have been reported by several UK producers and have resulted in losses of up to 10% of some crops, since affected specimens must be downgraded or discarded. The mean incidence of ISN on one UK farm was 0.3% of mushrooms harvested at the button stage and 5.6% of mushrooms harvested at the open, flat cap stage (J. L. Burden, unpublished data). When the fine line between profit and loss in the UK mushroom industry is considered, the importance of an understanding of this disease process becomes clear.

The symptoms of ISN take the form of a variable browning reaction in the centre of the mushroom stipe, and some specimens may also exhibit a slight browning of

internal cap tissue abutting the stipe core. As the mushrooms mature, affected tissues may collapse, in some instances leaving only a brown peg of dry tissue adhering to the inside of the cap. Progress of symptoms beyond this stage is not observed, suggesting that the disease is somewhat self-limiting. In all cases, damage is only revealed on harvest. Preliminary examination of diseased mushrooms revealed the presence of significant numbers of bacteria. This, and the fact that viral and fungal screens of diseased mushrooms were negative, led to the hypothesis that ISN is caused by bacterial infection (J. Newbury & J. F. Peberdy, unpublished data).

An earlier report on ISN of *A. bisporus* attributed the disease to *Pseudomonas fluorescens* (Richardson, 1993). This observation was based on a simple injection trial, but failed to present thorough evidence of the satisfaction of Koch's postulates, or that the disease could be initiated by this isolate under farm conditions. The phenotype of the presumptive agent was also not described, so that assignment to one of the diverse subdivisions of the *P. fluorescens* complex is impossible.

The aims of this study were to thoroughly investigate the microflora of mushrooms showing ISN symptoms and to

Abbreviation: ISN, internal stipe necrosis.

select organisms for Koch's postulate trials based on rational principles: namely, consistent association of bacterial species with the disease. Bacteriological media and methods were designed to differentiate *Pseudomonas* sub-species and strains and to select for non-pseudomonad bacteria. Significant problems may be encountered in the verification of Koch's postulates when conducting trials in an essentially non-sterile environment (normal mushroom cultivation). In this study, these problems were avoided by tracking applied strains using RFLPs as molecular markers. The study yielded an unexpected result, namely the recovery of the bacterium *Ewingella americana*, a first report of this organism from this source.

METHODS

Primary isolation, identification and screening for *E. americana*. Diseased mushrooms were regularly collected and analysed, between 1991 and 1993, from three UK farms, two situated in Selby, N. Yorks. (Farms 1 and 2) and one situated in Langford, Avon (Farm 3). During this period at least 70 individual symptomatic mushrooms were subjected to analysis. For quantitative bacteriology, mushroom stipes were aseptically dissected and symptomatic tissue removed, weighed and homogenized in 10 ml Maximum Recovery Diluent (MRD, Oxoid). Samples were then serially diluted in MRD, and plated on Luisetti (Luisetti *et al.*, 1972), MacConkey (Oxoid) and LB (Miller, 1972) agars. Cultures on the latter medium were incubated anaerobically at 28 °C and the others incubated aerobically at 28 °C, for 48 h. Colonies were counted, and distinct types subcultured for later identification. For long term storage, isolates were kept in 15% (v/v) glycerol at -80 °C. Presumptive *P. fluorescens* isolates were identified according to the probability matrix of Gennari & Dragotto (1992). Bacteria isolated from anaerobically incubated plates were subcultured aerobically on MacConkey agar and lactose-fermenting colonies identified using the API20E system (bioMérieux) according to the manufacturer's instructions. Selected strains were also identified by API50CHE (bioMérieux). All strains were screened for production of chitinase using the medium of Wirth & Wolf (1990) supplemented with 10 g Tryptone (Merck) l⁻¹ and 5 g Yeast Extract (Oxoid) l⁻¹. When incubated anaerobically, this medium could be used to select and differentiate presumptive *E. americana* in samples heavily contaminated with *P. fluorescens*. This technique was also adopted in qualitative tests where mushroom stipes were sampled by swab and plated out directly.

Experimental strains. The strain of *A. bisporus* used in Koch's postulate trials was the commercial strain Somycel 512, which was also commonly cultivated on the farms contributing diseased mushrooms during the primary sampling phase of this study. Bacterial isolates derived from diseased mushrooms and selected for re-inoculation tests were PI4, PI74, PI98, PI145 and PI180. Strain PI4 is a *P. fluorescens* biovar K isolate, which is unusual among mushroom isolates in that it produces a chitinase. The other strains were *E. americana* isolates randomly selected from the University of Nottingham collection of mushroom-stipe-necrosis-derived bacteria isolated during this study. Strain PI145 was originally isolated from a mushroom grown at Farm 3. The other *E. americana* isolates were from mushrooms harvested at Farms 1 and 2. The *E. americana* strains are now deposited in the National Collection of Plant Pathogenic Bacteria (NCPBB), Hatching Green, UK as with accession numbers NCPBB 3903, 3905, 3906 and 3908, respectively.

Escherichia coli strain XL1-Blue (Stratagene) was used in DNA manipulations.

Koch's postulate trials. Bacteria for re-inoculation trials were propagated by shaking at 28 °C for 18 h in M9 minimal salts broth (Gibco) supplemented with 10 g D-glucose l⁻¹. Flasks (250 ml; Pyrex conical) contained 100 ml medium. Such cultures contained a mean bacterial population of 3.7×10^8 c.f.u. ml⁻¹ (viable count). Experiments were conducted at a commercial mushroom farm using the shelf system of production. Following re-inoculation, trial beds were treated as in normal production with regard to watering, harvesting schedule and post-harvest sterilization (Fletcher *et al.*, 1989). Following pinning, beds were routinely chlorinated during watering. ISN symptoms on harvest were scored on a severity scale of 1-5 (least-most; defined in the legend to Table 3). Symptoms recorded with a rating of 3 and above corresponded to the typical range of ISN seen in natural infections, whereas symptoms with a rating of 1 or 2 were atypically mild but significantly discoloured compared to untreated mushrooms of the same variety.

Re-inoculation methods

(i) **Direct injection.** The method of Richardson (1993) was adapted so that 100 µl bacterial culture in M9 + glucose broth, containing approximately 3.7×10^8 viable cells, was injected with a 23G hypodermic needle into the stipe bases of 50 young mushrooms at the 'pin' stage, marked on the cap for later identification. Control treatments comprised batches of 50 mushrooms injected with M9 + glucose broth and 50 uninjected mushrooms.

A second injection trial was later set up using buffer-washed bacteria in order to eliminate nutrient and bacterial metabolite accumulation effects. Here, bacteria were grown as above, harvested by centrifugation and washed twice in sterile PBS (0.145 M NaCl, 0.15 M sodium phosphate; pH 6.5). Following resuspension in PBS, cells were serially diluted and each dilution injected into batches of 50 developing mushrooms as above. Controls consisted of 50 mushrooms injected with PBS alone and 50 untreated mushrooms. Diluted bacteria were enumerated by viable count. All mushrooms were harvested 7 d later and examined for symptoms and bacterial colonization as described above.

(ii) **Spray inoculation.** Bacterial cultures (1l), prepared as above, were evenly applied to a 1 m² plot of a mushroom bed at the stage when mycelium could be observed to have freshly colonized the surface of the casing layer. Mushrooms were picked at the button stage and 10% of the flush left to develop further to the 'flat' stage. All mushrooms from the test plots were inspected on harvest and symptomatic specimens examined bacteriologically.

RFLP analysis. Genomic DNA was prepared according to Pitcher *et al.* (1989). The *E. americana* 16S ribosomal RNA gene was amplified from strain PI98 by PCR according to Lane *et al.* (1985), and cloned using the pGEM-T vector (Promega). Following plasmid propagation in *E. coli* and extraction with the Quiaprep spin kit (Quiagen), 1 µg insert DNA was excised from the vector, purified using the GeneClean II kit (Bio 101) and labelled with biotin-14-dATP using the NEBlot Phototope (New England Biolabs) random primer labelling kit according to the manufacturer's instructions. Genomic DNA from applied and re-isolated strains (1.0 µg) was digested to completion with *Pvu*II and Southern blots set up according to Sambrook *et al.* (1989). Hybridization was carried out with the 16S rDNA probe at high stringency and signals detected using the Phototope detection kit (New England Biolabs) according to the manufacturer's instructions.

Confirmation of identity of ISN strains as *E. americana*. A filter DNA hybridization technique, adapted from that described by Jahnke (1994), was used to compare selected *E. americana* strains derived from stipe necrosis infections with phenotypically similar reference strains. The type strain of *E. americana*, ATCC 33852 (NCTC 12157), was obtained from the UK National Collection of Type Cultures (NCTC). Other reference strains were obtained from the UK National Collection of Industrial and Marine Bacteria (NCIMB). Phytopathogenic type strains were kindly provided by Dr D. E. Stead, NCPPB.

Test DNA (1 µg) was diluted with alkali transfer buffer (1.5 M NaCl, 0.25 M NaOH), boiled for 5 min and snap-chilled on ice. Samples were applied by vacuum to a prewetted nylon membrane (Hybond N+, Amersham), using a 96 well micro-filtration manifold (Schleicher & Schell, model SRC-96). Samples were applied in triplicate in a random arrangement, with several wells left blank for calibration purposes. DNA from strain PI98 was also applied in triplicate. Genomic DNA (1.5 µg) from strain PI98 was labelled with biotin-14-dATP as described above, yielding approximately 1.0 µg probe DNA. Hybridization was carried out at moderate stringency (68 °C, 0.1 × SSC, 1% (w/v) SDS; 1 × SSC is 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0), and chemiluminescent hybridization signals quantified using a phosphor imaging system (Bio-Rad, model GS-250), with a chemiluminescent detection cassette. Data from the imager were adjusted for background using readings from the blank wells. Relative hybridization was assessed by comparing data from test wells to that of fully homologous wells containing DNA from strain PI98.

Statistical analysis. Standard ANOVA techniques were used to analyse arcsin-transformed data from infection experiments. Means were compared using Fisher's protected least significant difference (LSD) test.

RESULTS AND DISCUSSION

Bacterial flora of ISN-affected mushrooms

The total number of bacteria isolated from ISN-affected mushrooms was usually within the range 10^8 – 10^{10} c.f.u. g fresh wt⁻¹, whereas normal internal stipe tissues were found to have a bacterial population of up to 10^4 c.f.u. g⁻¹. This control figure is lower than previously published accounts of the mushroom microflora (Doores *et al.*, 1986), but probably reflects the fact that in this study, the specimens examined were aseptically dissected, thereby avoiding gross surface contamination. All strains of *E. americana* examined, including ATCC 33852, were found to be chitinolytic. Interestingly, this property appears to be constitutive in these strains, since the enzyme has been found to accumulate in minimal broths (M9 + glucose) without the addition of chitin or mycelial preparations (Inglis, 1995). In the original description of *E. americana* (Grimont, 1983), chitinase production was recorded as negative. This may reflect a difference in the screening methodologies, since in this study we used a nutrient base, thereby allowing growth of strains unable to utilize the products of chitin digestion. The screening method adopted was found to be effective in selecting and differentiating *E. americana*, even in specimens heavily contaminated with *P. fluorescens*. The vast majority of fermentative, chitinolytic bacteria isolated from mushrooms selected using this technique were found in

Table 1. Reassociation of DNA from ISN strain PI98 with that of other ISN and reference (type) strains

Washes were at 68 °C in 0.1 × SSC. Signals were recorded as mean counts per hour, with fully homologous (PI98) counts averaging 2527.2. Background counts were 396.4.

Species/strain	Relative binding (%) ± SEM
ISN strain PI98 (NCPPB 3905)	100 ± 4.9
ISN strain PI180 (NCPPB 3908)	91.8 ± 5.3
ISN strain PI200 (NCPPB 3909)	88.7 ± 3.9
ISN strain PI74 (NCPPB 3903)	88.2 ± 4.1
ISN strain PI145 (NCPPB 3906)	87.6 ± 4.3
ISN strain PI166 (NCPPB 3907)	85.1 ± 0.6
ISN strain PI91 (NCPPB 3904)	80.1 ± 1.0
<i>Ewingella americana</i> ATCC 33852	88.8 ± 3.6
<i>Erwinia amylovora</i> NCPPB 683	22.6 ± 1.2
<i>Hafnia alvei</i> NCIMB 11999	22.0 ± 1.5
<i>Enterobacter cloacae</i> NCIMB 10101	21.9 ± 1.3
<i>Erwinia carotovora</i> subsp. <i>carotovora</i> NCPPB 312	20.6 ± 0.7

Table 2. Frequency of isolation in quantitative tests of bacterial species and sub-species from ISN-affected mushrooms

A total of 180 isolates from 70 individual mushroom samples were analysed between 1992 and 1993.

Species/sub-species	Frequency of isolation (%)
<i>E. americana</i>	93
<i>P. fluorescens</i> biovar K	53
<i>P. fluorescens</i> biovar IV-2	40
<i>P. fluorescens</i> biovar V-6	21
<i>P. putida</i> B	11
Unidentified enterics	9
<i>P. fluorescens</i> biovar III-1	9
<i>P. fluorescens</i> biovar III-2	4
<i>P. fluorescens</i> biovar II-1	4
<i>P. paucimobilis</i>	1
<i>P. fluorescens</i> biovar III-3	1

subsequent tests to be *E. americana*. DNA hybridization (Table 1) confirmed the identity of the ISN strains as *E. americana*, since values are well within the species break-point proposed by Wayne *et al.* (1987).

In quantitative samplings, *E. americana* was detectable in 93% of mushrooms showing ISN symptoms (Table 2). However, the lower limit of detection of the enumeration method adopted in this study is approximately 1×10^3 c.f.u. g⁻¹ so the absolute prevalence of this species in diseased mushrooms is possibly much higher. This is

Fig. 1. Bacteria recovered from ISN-affected mushrooms harvested from Farm 1 between 14 May 1992 and 23 July 1992. Results shown are mean bacterial numbers recovered from an average of six diseased mushrooms collected on the stated date.

supported by the results of qualitative sampling, where *E. americana* was found in 100 % of symptomatic mushrooms. *P. fluorescens* was also found in the majority of mushrooms sampled, but variation was great, both in terms of numbers of distinct biovars present and phenotypic diversity within those taxonomic groupings. Most ISN-affected stipes yielded a mixed bacterial flora, with *E. americana* usually being present in numbers of up to 10^6 c.f.u. g⁻¹ and *P. fluorescens* isolates typically being present in higher numbers of 10^7 – 10^9 c.f.u. g⁻¹ (Fig. 1). Some mushrooms analysed outside the main collection period, however, did yield much higher numbers of *E. americana*, often in pure culture (data not shown). None of the mushroom stipes displaying ISN symptoms examined had a total bacterial load of less than 10^7 c.f.u. g⁻¹. Frequently, two clearly distinct *P. fluorescens* types were co-dominant. Richardson (1993) also obtained *P. fluorescens* from ISN-affected mushrooms, but did not report on the biovar assignment of those strains, nor their phenotypic characteristics. In this study, mixed infection could present problems in the interpretation of Koch's postulates. However, the diverse taxonomic nature of the pseudomonads encountered in specimens and the lack of a single, consistently associated biovar probably implies secondary colonization of already damaged stipes. Some strains of the *P. fluorescens* complex are known to be pathogenic for plants. However, the taxonomy of this species in this regard has been discussed by Dye *et al.*

(1975), who argued that they exhibit 'phytopathogenic individuality', meaning that *P. fluorescens*, in terms of Koch's postulates, should not be regarded as a single species. Subsequent work has also indicated that the distinct *P. fluorescens* biovars commonly occupy discrete ecological niches (Champion *et al.*, 1980; Gennari & Dragotto, 1992). On the evidence of consistent association with ISN, therefore, *E. americana* strains were selected for further investigation in Koch's postulate experiments.

Koch's postulate trials

In the first injection trial (Table 3), treatment with *E. americana* produced ISN symptoms of typical severity (scale 3–5) in at least 40 % of mushrooms. Strain PI74 was found to produce a significantly greater incidence of typical ISN symptoms than the other *E. americana* strains tested for reasons that are currently unclear. Mushrooms injected with *P. fluorescens* were significantly less severely affected ($P < 0.001$), with only 6 % of injected mushrooms displaying symptoms in the typical range. The remaining 50 % of affected mushrooms in this treatment displayed atypically mild symptoms that did not match that seen in normal ISN disease, although these were significantly discoloured when compared to control (uninjected) stipes. *E. americana* was re-isolated in almost pure culture from all *E. americana*-injected mushrooms showing typical symptoms and was usually present in excess of

Table 3. Results of first injection trial

In each treatment, 50 mushrooms were injected with 100 µl bacterial culture or uninoculated M9 + glucose broth. ISN symptom severity was recorded on a scale of 1–5 (least–most), defined as follows: 1, trace straw-coloured discolouration, little longitudinal spread from cut base, atypical; 2, mild straw/ginger discolouration, spread < 30 % stipe axis, atypical; 3, light brown discolouration, spread < 50 % stipe axis, lower limit of typical range; 4, dark brown discolouration, spread > 50 % stipe axis, mild cavitation, mean of typical range; 5, dark brown discolouration spreading throughout the stipe axis, severe cavitation, upper limit of symptom range.

Strain applied	Total symptomatic mushrooms (ratings 1–5) (%) [*]	Mean symptom severity ± SEM	Mushrooms with rating > 3 (%) [*]
<i>P. fluorescens</i> PI4	56	1.86 ± 0.19	6
<i>E. americana</i> PI74	76	4.27 ± 0.07	70
<i>E. americana</i> PI98	40 ^a	4.21 ± 0.09	40 ^a
<i>E. americana</i> PI145	42 ^a	4.75 ± 0.09	42 ^a
<i>E. americana</i> PI180	44 ^a	4.53 ± 0.15	44 ^a
Control (M9 + glucose)	30	2.32 ± 0.21	16
Control (uninjected)	0	–	0

^{*} Within each column, values followed by the same letter are not significantly different according to Fisher's protected LSD test ($P < 0.001$). For ANOVA, asymptomatic mushrooms were given a rating of 0.

10^7 c.f.u. g^{-1} . These recovered strains were phenotypically identical to those applied when tested with the API50CHE system. In the group injected with *P. fluorescens*, bacteriological examination of the mildly affected specimens yielded variable numbers of the strain applied. In several cases, however, *E. americana* was also recovered in small numbers when screened, along with several distinct colonial types resembling *P. fluorescens* when cultured on Luisetti agar. The total *Pseudomonas* population in mildly affected mushrooms following this treatment was in the range 10^6 – 10^{10} c.f.u. g^{-1} . Mushrooms with symptoms in the typical range were all found to be colonized by *E. americana*.

Injection with sterile broth produced symptoms in 30 % of mushrooms but again, these were significantly less severely affected than the *E. americana*-treated groups. On bacteriological examination, specimens with a severity score of 3 and greater were also found to be colonized by *E. americana* at 10^4 – 10^9 c.f.u. g^{-1} , along with variable numbers of fluorescent pseudomonads. Specimens in the mildly affected group yielded between 10^6 and 10^{10} c.f.u. fluorescent pseudomonads g^{-1} , with *E. americana* being present in most samples in trace numbers. No ISN-affected mushrooms were found in the uninjected control population.

Typical disease symptoms were also produced in mushrooms from trial plots sprayed with *E. americana* (Table 4), although disease incidence was considerably less than that following injection. *E. americana* was re-isolated from 100 % of those mushrooms showing symptoms in the typical range (scale 3–5). Strain PI74 again demonstrated significantly greater virulence than other *E. americana*

isolates, which otherwise did not demonstrate significant differences in disease incidence or symptom severity ($P < 0.001$). Mushrooms from the plot treated with *P. fluorescens* isolate PI4, as in the injection trial, developed atypical symptoms and bacteriological examination also revealed colonization by *E. americana* in many of these mushrooms, along with mixed *P. fluorescens* strains. Total bacterial numbers in these specimens was in the range 10^7 – 10^{10} c.f.u. g^{-1} . Bacteria phenotypically identical to the applied pseudomonad (chitinase-positive) were not reliably recovered from these mushrooms. The plot treated with broth yielded several typically symptomatic mushrooms heavily colonized with *E. americana* at 10^7 – 10^9 c.f.u. g^{-1} , several of which were also colonized by several distinct colonial types of *P. fluorescens*. Fluorescent pseudomonads have been found to be ubiquitous in casing soils and colonized composts (Samson *et al.*, 1986; Doores *et al.*, 1986), and it is not surprising that miscellaneous strains should colonize damaged mushrooms. Mean symptom severities caused by broth and *P. fluorescens* treatments were significantly less than that caused by *E. americana* treatment ($P < 0.001$). The observation that the disease was almost completely suppressed at the second flush probably indicates that the causative bacteria are effectively inhibited by normal chlorination schedules. This may explain the observed level of occurrence of the disease on mushroom farms, with outbreaks possibly exacerbated by faulty hygiene or disinfection regimens. Chlorination may also have contributed to the low disease incidence following spray inoculation, but this may also have been caused by natural resistance to infection by undamaged *Agaricus* mycelium. Microbial interactions in the casing soil may also have

Table 4. Results of spray inoculation trial

Mushroom beds were sprayed with 1 l bacterial culture or uninoculated M9 + glucose broth. Average numbers of mushrooms (90% picked at 'button' stage; 10% picked later at 'flat' stage) harvested per m² plot were 256 for the first flush and 241 for the second flush. These values were consistent with the mean and variance of the yield in the total crop harvested from the same growing house as the test plots (data not shown). ISN symptom severity was recorded on a scale of 1–5 (least–most) as defined in the legend to Table 3.

Strain applied	Total symptomatic mushrooms (ratings 1–5) (%)*		Mean symptom severity $\pm$ SEM	Mushrooms in 1st flush with rating > 3 (%)*
	1st flush	2nd flush		
<i>P. fluorescens</i> PI4	14.8	0.5 ^a	1.83 $\pm$ 0.21	0
<i>E. americana</i> PI74	29.3	3.2	4.36 $\pm$ 0.07	25.3
<i>E. americana</i> PI98	11.5 ^a	0	4.12 $\pm$ 0.19	10.8 ^a
<i>E. americana</i> PI145	11.7 ^a	0	4.66 $\pm$ 0.15	11.7 ^a
<i>E. americana</i> PI180	11.8 ^a	0	4.65 $\pm$ 0.09	11.8 ^a
Control (M9 + glucose)	19.9	0.7 ^a	2.24 $\pm$ 0.19	5.3
Control (untreated)	0	0	–	0

* Within each column, values followed by the same letter are not significantly different according to Fisher's protected LSD test ($P < 0.001$). For ANOVA, asymptomatic mushrooms were given a rating of 0.

influenced the incidence of ISN in plots sprayed with bacteria. This is supported by the observation that no mushrooms were harvested with an ISN severity score of 3 and above from the plot sprayed with *P. fluorescens*, compared to 6.3% of mushrooms from the plot sprayed with sterile broth. Further co-inoculation experiments with *E. americana* and other micro-organisms collected from casing soils and composts could be useful in determining this possible influence on ISN development, which could be either positive or negative. The timing of bacterial inoculation relative to the stage of mushroom mycelial development is likely to be similarly important.

In the first injection and the spray trials, inocula were deliberately large, since there is no data currently available on the rates of survival of *E. americana* in either casing soils and in mushroom tissues. In a previous study (Wong & Preece, 1982), population levels of *Pseudomonas tolaasii* were found to decline rapidly in casing soils, and at least 6×10^7 c.f.u. ml⁻¹ were necessary to produce the typical lesions of brown blotch disease. However, to eliminate the stimulatory effects of nutrient addition to the system, and to remove possible antagonistic metabolites such as chitinase, it was necessary to conduct an inoculation trial using washed cells. Additionally, the effect of inoculum size was investigated (Table 5). Injection with washed cells of *E. americana* strain PI145 was found to cause a much higher disease incidence than cells remaining in the culture broth (Table 3). This may have been due to the removal of accumulated bacterial inhibitors from the inoculum, or the lack of nutrients in the inoculum that may stimulate potentially inhibitory competitor micro-organisms. High levels of disease incidence were repeated down to the smallest inoculum used, although severity of symptoms began to decline when less than 3.7×10^4 cells

were injected. Significantly, the buffer control in this trial produced very mild symptoms in 8% of injected mushrooms.

The variability in the relative population levels of *E. americana* and *P. fluorescens* following artificial inoculation, and of that found in natural infections, probably indicates that total bacterial numbers in infected stipes is a major contributing factor in determining pathogenesis. Asymptomatic mushrooms following injection with either *E. americana* or *P. fluorescens* were found to contain a total bacterial population of less than 10^7 c.f.u. g⁻¹, whereas symptomatic mushrooms were usually found to harbour a bacterial population substantially in excess of this figure. However, in these trials *E. americana* was found to be essential for typical symptom development, whether in pure or mixed culture. In natural infections, we propose the model that *E. americana* is likely to initially colonize the internal stipe of *A. bisporus* and cause cellular damage, possibly as a result of chitinase production. This is supported by the finding of Mol & Wessels (1990) that the stipe cells of *A. bisporus* are particularly vulnerable to chitinase digestion as compared to substrate hyphae and non-expanding tissues. Following such damage and resultant nutrient leakage, other saprophytic bacteria such as *P. fluorescens*, naturally present in small numbers within mushroom tissues, may proliferate, thereby contributing to symptom development.

Since little phenotypic variation was discernible in *E. americana* strains, it was necessary to use molecular markers to detect strain boundaries, and to prove that strains re-isolated following artificial infection were identical, thereby confirming Koch's postulates. RFLP analysis clearly differentiated the inoculated strains, and

Table 5. Results of second injection trial

In each treatment, 50 mushrooms were injected with 100 µl washed *E. americana* PI145 cells in PBS. ISN symptom severity was recorded on a scale of 1–5 (least–most), as defined in the legend to Table 3.

No. bacteria applied (log ₁₀ c.f.u.)	Total symptomatic mushrooms (ratings 1–5) (%)*	Mean symptom severity ± SEM	Mushrooms with rating > 3 (%)*
8	100 ^a	4.28 ± 0.11	100 ^a
7	100 ^a	4.02 ± 0.11	100 ^a
6	88	3.86 ± 0.09	88
5	98 ^a	4.52 ± 0.08	98 ^a
4	82	4.77 ± 0.06	82
3	100 ^a	4.01 ± 0.11	100 ^a
2	92 ^a	3.15 ± 0.10	54
Control (buffer)	8	1.49 ± 0.25	0
Control (uninjected)	0	–	0

* Within each column, values followed by the same letter are not significantly different according to Fisher's protected LSD test ($P < 0.001$). For ANOVA, asymptomatic mushrooms were given a rating of 0.

Fig. 2. RFLP analysis of *E. americana* test strains and strains recovered from infection experiments. Genomic DNA was digested with *PvuII* and hybridized overnight with an *E. americana* 16S rRNA gene probe. Washes were at 68 °C in 0.1 × SSC, 1% SDS. Lanes: 1, pre-biotinylated mixture of *HindIII*-digested lambda DNA and *HaeIII*-digested ϕ X174 DNA; 2–5, *E. americana* ISN strains PI74, PI98, PI145 and PI180, respectively; 6, *E. americana* ATCC 33852; 7–8, strains reisolated from first and second flushes, respectively, of mushrooms injected with PI74; 9–15, strains recovered from diseased mushrooms harvested from plots sprayed with PI98, PI145 and PI180 (9–11, respectively) and with M9 broth + glucose (12–15); 16, chitinolytic enteric strain isolated from peat sample.

these profiles were found to match those of the majority of strains recovered from individual infection experiments (Fig. 2). A clear distinction could be made between the RFLP profiles of test strains and those of *E. americana* strains recovered from diseased mushrooms harvested from the plots sprayed with broth. At least five novel

RFLP types were identified among this latter population, which were collected from mushrooms grown in a plot of just 1 m². This is further evidence for the supposition that *E. americana* was endemic at the time of the inoculation experiments, and that a considerable diversity of strains was present in the farm environment.

Attempts to recover *E. americana* from uncolonized casing soils and compost were unsuccessful (data not shown). This may be due to low numbers being naturally present, as is the case with *P. tolaasii*, which is thought to be stimulated by the presence of living *A. bisporus* mycelium (Wong & Preece, 1980). Further enrichment experiments are necessary to determine the primary sources of this bacterium in the farm environment. Previously, *E. americana* was only infrequently isolated from human clinical specimens, where it usually takes the form of an opportunistic colonizer (Grimont *et al.*, 1983). Contamination by farm workers carrying *E. americana* is, therefore, a potential mode of introduction. Among clinical isolates, several have been recovered from stool specimens, suggesting that contamination of the farm environment by sewage may also be a possible mode of introduction of this species. Recently, *E. americana* has also been recovered from the guts of molluscs (Müller *et al.*, 1995), thus indicating that this species may have a much wider environmental distribution than once suspected. *A. bisporus* is a new host for *E. americana*. It is therefore of interest to understand the basic mechanisms that cause this bacterium to assume a pathogenic role in mushrooms. We propose to address this question in future work. A deeper understanding of the disease process may also enable us to predict the potential of other bacteria associated with mushroom culture to cause disease in *A. bisporus*, and to design cultivation practices that reduce the chances of potentially serious diseases emerging.

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