Internal Stipe Necrosis of *Agaricus bisporus* - Etiology and Molecular Genetic Studies

by
Peter Ward Inglis
BSc., MSc.

Thesis submitted to the University of Nottingham
for the Degree of Doctor of Philosophy
October, 1995

To Mum & Dad

Contents

Acknowlegments...i

Abstract...ii

Abbreviations....iv

CHAPTER 1: General Introduction...**1**

Internal stipe necrosis - occurrence and impact......................................2
The bacterial diseases of *Agaricus bisporus* and other edible mushrooms:
Brown blotch 5
Ginger blotch 11
Mummy disease 12
Drippy gill and related disorders 14
Bacterial pit...15
Diseases of other *Agaricus* species...15
Bacterial diseases of non- *Agaricus* mushrooms....................................16
The "normal" bacterial flora of cultivated *Agaricus* mushrooms and growing media 17
The physiological response of *A. bisporus* to infection..........................21
Virulence determinants in bacterial diseases of mushrooms...................25
Objectives of this research...28

CHAPTER 2: Bacteriological analysis of internal stipe necrosis affected Mushrooms..**29**

INTRODUCTION 29
MATERIALS AND METHODS 31
Diseased mushroom collection 31
Specimen preparation and primary isolation of bacteria 31
Preliminary identification of fermentative bacteria 32
Identification of presumptive *Pseudomonas* species and biovars...33
Miscellaneous characterisation tests:
Test for gelatinase 33
White line in agar test for *P. tolaasii* 35
Production of chitinase 35
Production of lipase (Tween 80 esterase) 35
Production of siderophores 35
Haemolysis..36

Histochemical staining of tissue blots for mushroom tyrosinase 36
RESULTS 37
DISCUSSION 41

CHAPTER 3: The application of Koch's Postulates to internal stipe necrosis of *Agaricus bisporus* 46

INTRODUCTION 46
Molecular techniques in bacterial epidemiology 47
MATERIALS AND METHODS 51
Strains and vectors 51
Media and growth conditions 51
Pathogenicity trials:
Reinoculation methods
i. Direct injection 52
ii. Spray inoculation 52
Restriction fragment length polymorphism (RFLP) analysis of re-isolates:
Preparation of bacterial genomic DNA 52
Agarose gel electrophoresis 53
Amplification of the 16s ribosomal RNA gene from enteric cluster strain PI98 by the polymerase chain reaction (PCR) 54
Cloning of PCR products 54
Transformation of *E. coli* 55
Preparation of biotinylated probe DNA 56
Southern Blotting:
Digestion and electrophoresis of bacterial genomic DNA 56
Transfer and immobilisation of DNA onto Nylon membranes 56
Hybridisation with a biotinylated 16s rDNA probe 57
Stringency washing and detection of DNA hybrids 57
RESULTS 58
Molecular fingerprinting and confirmation of Koch's postulates
DISCUSSION 65

CHAPTER 4: Taxonomy of the stipe necrosis causative agent 69

INTRODUCTION 69
Molecular techniques applied to bacterial taxonomy 70
MATERIALS AND METHODS 72
Physiological profile of the stipe necrosis agent. 72
Analysis of cellular fatty acid content of ISN strain PI98 72
Antibiotic sensitivity testing 73

Electron microscopy of stipe necrosis strain PI98 (Negative stain). 73
Sequencing of the 16S ribosomal RNA gene from stipe necrosis strain PI98:
Preparation of Single stranded DNA for sequencing. 74
DNA sequencing. 75
Determination of DNA hybridisation values:
Preparation of a genomic probe. 75
Immobilisation of test DNA's. 76
Hybridisation and quantitation. 76
RESULTS 77
FAME profile of ISN strain PI98 77
Phenotypic characterisation of ISN strains 78
Electron microscopy of ISN strain PI98 78
16S rRNA gene sequencing 81
Genomic DNA Hybridisation analysis 84
DISCUSSION 86

CHAPTER 5: Purification and some properties of a chitinase from *Ewingella americana* 90

INTRODUCTION 90
Enzymatic degradation of chitin 91
MATERIALS AND METHODS 93
Preparation of chitin derivatives:
Ball milled native chitin 93
Colloidal (swollen) chitin 93
Glycol chitin 94
Carboxymethylated (CM) chitin 94
Dye labelling of CM chitin 95
Plate screens for chitinase induction 95
Plate test for lysozyme activity 95
Preparation of crude bacterial protein:
Strains and fermentation 95
Partial purification of chitin binding fractions by batch affinity chromatography 96
Hydrophobic interaction chromatography (HIC) 97
Protein quantification 97
Chitinase assay 97
Activity of chitinase against chromogenic soluble chitin derivatives 98
Polyacrylamide gel electrophoresis under denaturing conditions (SDS -PAGE) 98
Detection of chitinolytic activity in polyacrylamide gels 99
Characterisation of *E. americana* chitinase activity:

Determination of temperature optimum for chitinase activity 99
Determination of thermal stability of chitinase 100
Determination of optimal pH for chitinase activity 100
Determination of pH stability of chitinase.......................100
Effect of ionic strength on chitinase activity 100
Effect of metal ions on chitinase activity.........................100
Effect of EDTA, SDS and urea on chitinase activity..101
RESULTS 101
Regulation of chitinase and lysozyme activity 101
Chitinase purification..101
Classification of chitinase activity...103
Determination of multiplicity of chitinolytic enzymes in *E. americana*..103
Biochemical characterisation of *E. americana* PI98 chitinase 104
DISCUSSION..109

CHAPTER 6: Cloning and sequence analysis of a chitinase gene from *Ewingella americana* **114**

INTRODUCTION...114
MATERIALS AND METHODS 117
Construction of a plasmid / genomic DNA library for *E. americana* Strain PI98 (=NCPPB 3905).....................................117
Transformation of *E. coli* and selection of recombinants 117
Screening transformants for expression of chitinase..................117
Preparation of plasmid DNA 118
Subcloning, preparation of deletion mutants and sequencing strategy...118
Construction of unidirectional nested DNA deletions with exonuclease III and mung bean nuclease....................................118
DNA Sequencing 119
Assembly of chitinase sequence data 120
Analysis of sequence data..120
Determination of chitinase gene copy number 120
RESULTS 120
Library screening and selection of transformants........................120
Copy number of the cloned chitinase gene in the *E. americana* genome...121
Restriction mapping and deletion analysis 122
Nucleotide sequence analysis 125
Analysis of the deduced amino acid sequence of ChiA 126
DISCUSSION..131

CHAPTER 7: General Conclusions...**139**

Appendix: Abbreviations for amino acids...**145**

References **146**

Acknowlegments

I would like to express my thanks to Professor John Peberdy for his patient supervision and kindness during the course of this studentship. I am also grateful to Dr. John Burden for supplies of mushrooms (for study and for eating) and for a constantly warm welcome at Selby.

Thanks are also due to Dr. Liz Sockett and Dr. John Lucas for invaluable discussions and help. To Dr. Deepan Shah, for training in DNA sequencing, and to Dr. David Stead for his generous gift of reference bacterial strains.

I am also grateful to the clerical and technical staffs and to the photographic unit of the Department of Life Science. A special thank you must go to David West, who was always game for a laugh.

Special thanks must go the all the people who worked with me in lab 116 and became good friends: Songer Chen, Eddie Deane, Paul Dyer, Marcia Furlaneto, Andrea Edwards, Jo Hothersall, Jian-Hua Jia (Miss), Cas (Kahzi) Kramer, Gordon Lowery, Antonella Pane, Mike Papaikonomou, Zhu Ping, Sigit Pratigyno, Sonja Trojahn.

Most thanks must go to my parents, Violet and Stanley Inglis, for their constant support; and finally, to Cléria Valadares, - my most precious discovery.

I gratefully acknowledge the financial support of the BBSRC (formally SERC) and Middlebrook Mushrooms Ltd. for provision of a Postgraduate CASE studentship

Abstract

The button mushroom, *Agaricus bisporus* is the most popular mushroom in cultivation worldwide, and is the most valuable protected crop in the UK, with an estimated wholesale value exceeding £250 million. In 1991 a new disease emerged in mushroom crops in the UK, called Internal Stipe Necrosis (ISN). Crop losses due to this disease may reach 10 %, since affected mushrooms must be downgraded or discarded. Symptoms take the form of a variable browning reaction in the central region of the mushroom stipe, which may also demonstrate varying degrees of internal collapse.

During an exhaustive study of ISN over the past 3 years, it was found that an unusual enteric bacterium was consistently associated with the disease, along with diverse members of the *Pseudomonas fluorescens* complex, which probably represent secondary colonisers. Several strains of the enteric bacterium reproduced ISN symptoms in trials in which mushrooms were injected with bacteria and in trials where bacteria were sprayed onto otherwise normal mushroom beds. Isolates collected from deliberate infection experiments were shown to be identical to the applied strains by the use of restriction fragment length polymorphism (RFLP) studies, using a cloned 16s rRNA gene isolated from a representative strain of the enteric bacteria. These bacteria therefore appear to satisfy Koch's Postulates as the causative agent of ISN.

Conventional biochemical profiles identified the ISN causative agent as *Ewingella americana*, an unusual species previously unknown in mushrooms or their growing environment. This identification was confirmed by genomic DNA hybridisation using a range of reference strains taxonomically related to and including *E. americana*.

Evidence presented suggests that *E. americana* produces a single endo-acting chitinase. The significance of this enzyme in ISN pathogenesis is discussed. This 33 kDa enzyme has been purified by hydrophobic interaction chromatography and the encoding gene cloned and expressed in *E. coli.* Sequence analysis of this gene (designated *chiA*) revealed an open reading frame of 921 bp, with a deduced peptide size corresponding closely to the size of the purified enzyme. The deduced amino acid sequence was most similar to the chitinase II of *Aeromonas* sp. No. 10S-24 and, to a lesser extent, the chitinase of *Saccharopolyspora erythraeus*. Alignment with other chitinases, however, revealed very low homology with the exception of two conserved motifs in the catalytic domain of these enzymes. The *E. americana* sequence also lacks the chitin binding and Type III fibronectin homology units common to many bacterial chitinases. Deletion of a conserved motif, which has previously been implicated as forming the active site of chitinases, produced a product retaining significant chitinolytic activity. Such evidence may lead to a reappraisal of the significance of this motif in catalysis.

Abbreviations

bp	base pair
BSA	bovine serum albumin
cfu	colony forming unit
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside-triphosphate (dATP, dCTP, dGTP, dITP, dTTP, dUTP)
DTT	dithiothreitol
EDTA	ethylene-diamine-tetraacetic acid
g	gram; standard acceleration due to gravity
h	hour
ISN	internal stipe necrosis
IPTG	isopropyl-β-thiogalactopyranoside
kb	kilobase
min	minute
ONPG	*ortho*-nitrophenyl-β-*D*-galactopyranoside
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
rRNA	ribosomal RNA
R.H.	relative humidity
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl-sulphate
s	second; Svedberg units
SSC	saline, sodium citrate buffer
TBE	Tris, boric acid, EDTA buffer
Tris	Tris (hydroxymethyl) aminomethane
U	unit
UV	ultraviolet
v/v	volume/volume ratio
w/v	weight/volume ratio
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

CHAPTER 1
General Introduction

Agaricus bisporus (Lange) Imbach, the white button mushroom; syn. *A. brunnescens* Peck, has been in cultivation since 1550 (Poppe, 1978). Only the last 50 years, however, has seen the dramatic rise in the value of this crop, along with a more recent expansion of world-wide interest in industrial scale cultivation of this, and other, more exotic mushroom species; *A. bisporus,* however, remains the most widely grown mushroom (Table 1).

Table 1. World production of major edible mushrooms for 1991. Adapted from Chang (1993).

Unit: (metric ton x 1000)

Species	Common Name	Fresh wt.	%
Agaricus bisporus / bitorquis	Button mushroom	1590	37.9
Pleurotus spp.	Oyster mushrooms	917	21.5
Lentinula edodes	Shiitake or oak mushroom	526	12.3
Auricularia spp.	Wood-ear	465	10.9
Volvariella volvacea	Straw or Chinese mushroom	253	5.9
Flammulina velutipes	Winter mushroom	187	4.4
Tremella fuciformis	White jelly fungus or "Silver Ear"	140	3.3
Others		162.4	3.8
Total		4273	100

Fifty years ago, yields of *A. bisporus* were less than 5kg m^{-2}, whereas today, production levels at least ten times greater can be expected. Additionally, the duration of the harvest has been shortened from 12 weeks to 4 weeks (Chang, 1993). Such advances have largely been accomplished by optimisation of the composting and growing processes (Figure 1). The market currently expects a uniform and blemish-free product that the intensive production techniques now used attempt to provide. However, a consequence of such intensive cultivation methods is the potential for disease and serious crop losses. Mushrooms are the most valuable protected crop in the UK, and have an estimated world-wide value probably in excess of £1 billion (Grewal *et al*., 1994). When we consider the potential for economic loss associated with disorders affecting mushroom yield or quality the importance of an understanding of disease processes becomes clear.

Internal stipe necrosis - occurrence and impact

Since 1991, internal stipe necrosis (ISN) has been recognised as an emerging disease within the UK 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 (J.L. Burden, unpublished data). The symptoms of internal stipe necrosis (Figure 2) take the form of a variable browning reaction in the centre of the mushroom stipe and there may also be 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. In all cases, damage is only revealed on harvest. Another apparent characteristic is the sporadic nature of the disease, with no obvious patterns existing in distribution, in both time and space.

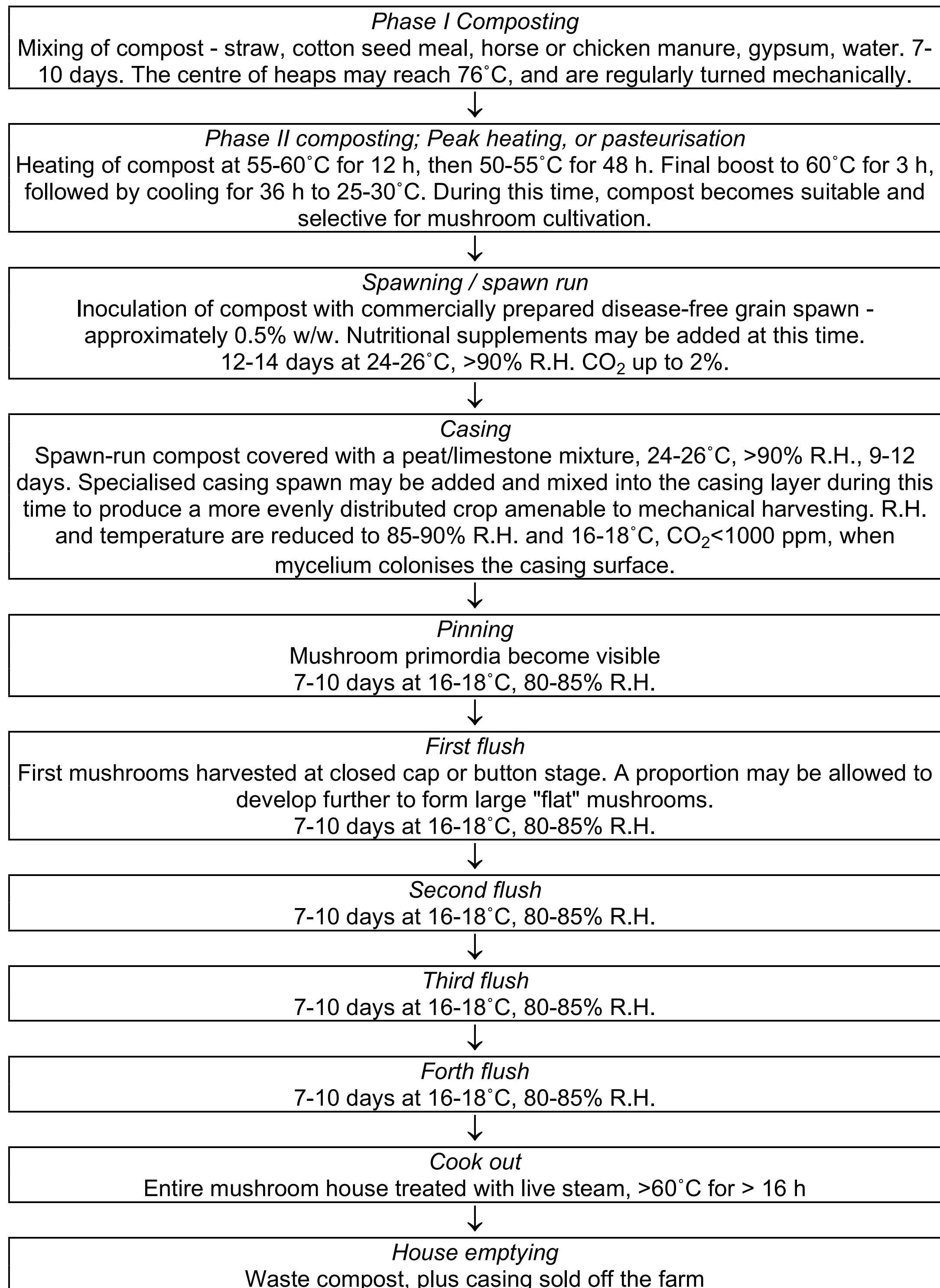
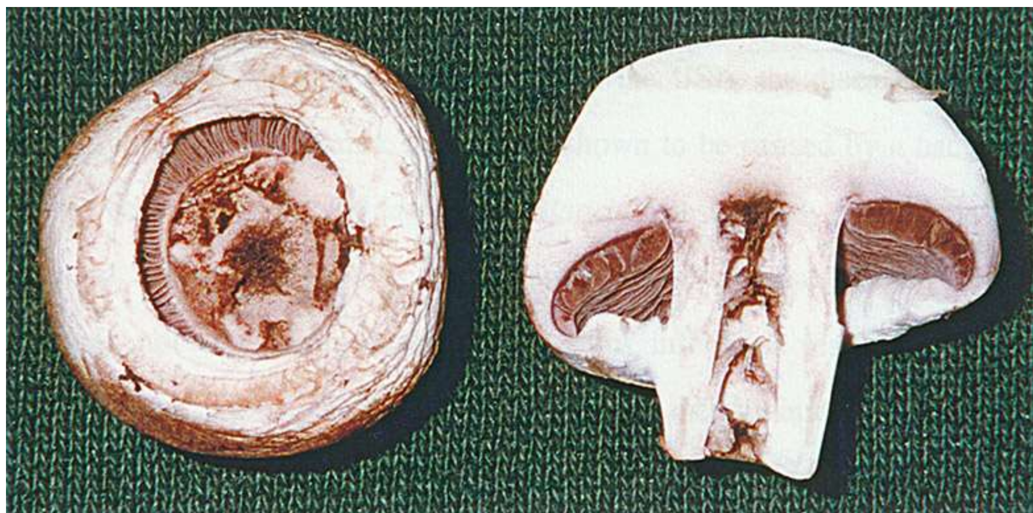


Figure 1. Generalised flow diagram of the processes of modern commercial *Agaricus* mushroom production. Adapted from Wong & Preece (1982) and Fletcher *et al.* (1989).

Outbreaks can appear at any stage of the cropping cycle, and appear to be randomly distributed on the mushroom beds (J.L. Burden, unpublished data). Preliminary examination of tissue from diseased regions of mushrooms showing these symptoms revealed the presence of significant numbers of bacteria. This, and the fact that viral and fungal screens were negative, led to the hypothesis that mushroom stipe necrosis is caused by bacterial infection (J.A. Newbury & J.F. Peberdy, unpublished data; J.L. Burden, personal communication).



Actual size

Figure 2. Internal stipe necrosis of *Agaricus bisporus* - typical symptoms.

During the course of this study, a report was published by Richardson (1993), implicating *P. fluorescens* as the causative agent of ISN. A distinction was also made with the random internal browning of mushrooms, caused by sciarid fly damage, which is not solely restricted to the longitudinal axis of the mushroom. However, the bacteriological evidence presented on true bacterial ISN involved a sampling of only 5 affected stipes. Bacteria were identified as *Pseudomonas*

fluorescens, but no attempt was made to place this strain into one of the many sub-divisions of this diverse assemblage, or to describe its phenotype. Injection of 12 developing sporophores with 0.5 ml of a heavy bacterial inoculum in nutrient broth, produced symptoms in 7 mushrooms. Such data does not indicate that Koch's postulates have been fully satisfied in this case, or that the strain of *P. fluorescens* could cause disease under normal farm conditions, during natural mushroom development.

The bacterial diseases of Agaricus bisporus and other edible mushrooms:

Brown blotch

Brown blotch was the first bacterial disease to be described in *A. bisporus* (Tolaas, 1915). Following the first report in the USA, the disease was soon recognised in the UK (Paine, 1919), and shown to be caused by a bacterium later to be renamed as *Pseudomonas tolaasii*. Subsequently, the disease has appeared wherever *Agaricus* mushrooms are grown (Cutri *et al.,* 1984). Although not completely destructive, serious infections result in severely discoloured mushroom caps that are unmarketable. Mildly infected mushrooms may still be useful for certain processed products, such as canning and soup manufacture, but are severely reduced in value for the grower. Owing to its prevalence and potential for economic loss, brown blotch is generally the most serious of the bacterial diseases of mushrooms (Fletcher *et al.,* 1989), and has necessitated a requirement for routine prophylactic chlorination of water applied to mushroom beds. It has been estimated that the disease, and necessary preventative measures, are responsible for economic losses of approximately 20% in most crops (Fermor, 1986).

The problems associated with brown blotch have stimulated considerable research interest into the biology of the disease and the causative organism. Identification of *P. tolaasii* was formally dependant on extensive and laborious

biochemical testing and pathogenicity assays (Gandy, 1968). Identification was made difficult by the close taxonomic relationship of *P. tolaasii* with the allied *P. fluorescens* species complex, which are also ubiquitous on mushroom caps and growing media (Unsworth & Preece, 1980; Zarkower *et al.,* 1984; Vantomme *et al.,* 1989). A breakthrough was made by Wong & Preece (1979), who identified a unique reaction between cultures of *P. tolaasii* and another *P. fluorescens* variant, now informally known as *Pseudomonas reactans*. The latter strains may be used as indicators in a plate assay, called the white-line-in-agar test, to positively identify the brown blotch agent. Such a simple test thus enhanced the prospects for further large-scale epidemiological investigations. Primary sources of *P. tolaasii* on a UK farm were found to be the peat and limestone making up the casing layer (Wong & Preece, 1980). Secondary to this, diseased or colonised *Agaricus* mycelium constituted a reservoir of infection that could then be spread to other beds or growing houses, via a variety of potential vectors. These included the hands, shoes and implements of the farm workers, dust and infected mushroom spores in the air of the houses and sciarid flies. Such data indicated the need for an awareness of the necessity of strict hygiene and infection control measures in such situations. Artificial inoculation experiments lead to the discovery that a relatively large bacterial inoculum was necessary to produce disease lesions on mushrooms; at least 6×10^7 cfu ml^{-1} being necessary to produce a blotch lesion when bacteria were applied directly to mushroom caps (Wong & Preece, 1982). However, when conditions favoured *P. tolaasii* proliferation, such as the retention of water films on the mushroom caps caused by overhead watering or condensation, much smaller inocula were necessary, and previously self-contained micro-lesions on cap surfaces were found to enlarge considerably. Although persisting in *Agaricus* mycelium colonised casing layers, *P. tolaasii* became almost undetectable in uncolonised casing following inoculation with as much as 6×10^8 cfu ml^{-1}. In the case of

colonised beds and mushroom caps, recovery rates following artificial inoculation were between 0.2 and 16.0%.

Chlorination of water applied to mushroom beds is currently the only generally approved method of *P. tolaasii* control in the UK. This is usually applied as a solution of sodium hypochlorite, but is rapidly inactivated when in contact with organic matter. Thus, the effectiveness of this disinfectant is severely limited when applied to mushroom beds (Royse & Weust, 1980; Wong & Preece, 1985a; Fletcher *et al.,* 1989). Alternatives have included more stable chlorine compounds such as chlorine dioxide. This requires the use of 50 µg ml^{-1} solutions for effective control, which is substantially above currently allowed limits. Numerous alternatives to chlorine have been investigated (Munjal *et al.,* 1987; Vantomme *et al.,* 1989), but some of these, including mercuric salts and antibiotics used in human disease therapy would never be acceptable. Wong & Preece (1985b) investigated the bactericide, Bronopol (2 bromo-2-nitropropane-1,3-diol), which was found to have excellent anti-pseudomonad properties, and effectively suppressed blotch disease in experimental infections. First investigated by Vantomme *et al.* (1989), and more recently and extensively by Geels (1995), the antibiotic kasugamycin (Hokko Chemical Industry Co., Ltd, Tokyo, Japan) shows promise in *P. tolaasii* control. The chemical is biodegradable to harmless oxalic acid and is not related to, or shows any cross-resistance to any human or veterinary antibiotic currently in use. A 1% solution applied following artificial infection of mushroom beds with a high inoculum during the first flush resulted in effective control during the second flush. Similar infected plots treated once with a sodium hypochlorite solution demonstrated similar disease rates to untreated controls. In Japan, kasugamycin has proven to be effective in bacterial disease control on a wide range of crops and shows little or no persistence.

Biological control may be an interesting alternative to chemical control measures for *P. tolaasii* (Nair & Fahy, 1972) and has been applied commercially (Nair & Fahy, 1976). Munjal *et al.* (1987) and Fermor & Lynch (1988) screened for antagonistic bacteria, and found strains of *Pseudomonas putida* and *P. fluorescens* biovar V that demonstrated significant suppression of disease. Henry *et al.* (1991) investigated the role of iron scavenging siderophores in biocontol of *P. tolaasii*. The most effective fluorescent pseudomonad screened suppressed disease by 55%, but it was found that antibiotic production by antagonists was probably more significant as a mode of action than siderophore production. This may be due to cross-feeding of siderophores that is known to occur between many bacteria, including several *Enterobacteriaceae* and between *Pseudomonas* species (Rabsch & Winkelmann, 1991).

The virulence of *P. tolaasii* is thought to be largely a consequence of the production of a toxin, known as tolaasin (Nair & Fahy, 1973). This toxin was shown to be a polypeptide, synonymous with the diffusable principle responsible for the white-line-in-agar test with *P. reactans* (Peng, 1986). Further work on the spectrum of activity and mode of action of tolaasin, showed the substance to be an ion channel-forming lipodepsipeptide (Brodey *et al*., 1991; Rainey *et al*., 1991). The substance, produced during the exponential and stationary growth phase, was found to partition into membranes and could cause lysis of erythrocytes. It was also found that the haemolysis caused by tolaasin could be prevented by the addition of osmotic stabilisers. Tolaasin was also shown to cause disruption of the plasma membranes of a wide range of basidiomycetes, and was also active against Gram-positive bacteria and plant cells. Gram-negative bacteria were not susceptible to tolaasin, except in the presence of sub-inhibitory concentrations of polymixin B; a treatment to which *P. tolaasii* remained resistant. The *P. tolaasii* toxin was shown to be exported as a complex of 2 active fractions; Tol I and Tol II, the latter of which is

the 1975 Da tolaasin *sensu stricto*. The toxin was shown to have a partially cyclic construction and proved to possess an extremely unusual left-handed α-helical structure with hydrophilic and hydrophobic domains, which may be responsible for its membrane targeting characteristics (Nutkins *et al*., 1991; Morticeshire-Smith *et al*., 1991). At around the same time, a cyclical peptide structure for the white-line indicating factor from *P. reactans* was also proposed (Hans *et al*., 1992), although it had already been described by Peng (1986) as a secondary amide. Tolaasin was subsequently shown to possess significant surface-active properties (Hutchison & Johnstone, 1993), which could also be related to the biological activity of the toxin, as well as the tendency of the bacteria to form spreading colonies on solid media.

Tolaasin was found to be synthesised as a result of the activities of 3 high molecular weight proteins (TL1, 464 kDa; TL2, 440 kDa; TL3, 435 kDa), which may function as tolaasin synthetases (Rainey *et al*., 1993). Such a mode of synthesis may explain the unusual structure of the mature toxin. There is also evidence to suggest that TL1 is membrane associated and may have a role in toxin secretion.

Phenotypic variation is a phenomenon common to many bacteria when grown for extended periods on solid media and may take the form of the development of sectors, morphologically distinct from the rest of the colony. Another type of variation is the instability of the margins of old colonies, where again, morphologically distinct, usually more translucent and spreading variants grow out from the original colony. Such phenomena have been reported in *P. tolaasii*, where smooth and rough forms of the bacterium were noticed (Oliver *et al*., 1978). The 2 forms, as well as being morphologically distinct, also varied in pathogenicity, so that the smooth form was pathogenic to mushrooms, was positive for the white-line test and toxin production, but was non-fluorescent; while the rough form was non-pathogenic, did not produce toxin and produced a greenish-yellow fluorescent pigment (Cutri *et al.,* 1984). However, in other conventional

biochemical tests, the 2 forms were indistinguishable. The smooth form had a tendency to convert to the rough form at a high frequency, but this could be reduced by growing the bacteria on a medium containing cetrimide, which was found to differentially inhibit the rough variant. Repeated sub-culture increased the stability of the rough form, but a stable smooth form was not obtained. Variation between different *P. tolaasii* strains was recorded, however, and a smooth form was recognised that remained stable for up to 10 days, while other strains became destabilised after 2-3 days incubation. No reversion of the rough form to the smooth was recorded. Various explanations for this phenomenon were proposed, including spontaneous mutation, phase variation and loss of extrachromosomal DNA. In addition to the above characters, phenotypic variants of *P. tolaasii* were found to display differences in chemotactic response (Grewal & Rainey, 1991) and ability to adhere (Rainey, 1991b) to mushroom mycelium; so that variants (rough-forms) displayed a stronger chemotactic response, but had less ability to adhere. These differences were suggested as a mechanism by which the bacterium survives nutrient depleted conditions, and could be adaptations to different environments. Later work showed that of the 2 classes of phenotypic variant in *P. tolaasii*, the sector-produced type remained stably converted, whereas that produced from unstable margins reverted to the wild-type morphology on sub-culture (Grewal *et al*., 1994). In this work, a cosmid library of wild-type *P. tolaasii* genomic DNA was successfully used to complement all of the changes in the stable variant form. This DNA was sub-cloned and a single 2.73 kb open reading frame, designated *pheN,* identified that could cause reversion of rough forms. Disruption of this wild-type gene in the smooth form caused phenotypic conversion identical to the conversion seen in sectors. Sequence analysis of the *pheN* gene revealed strong homology to several proteins comprising certain bacterial two-component sensor regulator systems, the closest of which was 87.5% homology to the *lemA* gene of *P. syringae*

pv. syringae. It is thought that the *pheN* product exists in a transmembrane state, with the N-terminal sensor domain in the periplasm. A currently unknown environmental stimulus interacts with the sensor and signals are then transmitted to the carboxy-terminus, where another gene product is likely to be involved in the regulation of transcription of the genes responsible for the phenotypic traits affected. The change that brings about sectoring appears to be a site-specific, natural insertion of a 665 bp DNA fragment into the *pheN* gene. Unstable margin phenotypic variants do not display such insertions, as is the case with sector revertants which appear to spontaneously lose their insertion sequence. The former class of variant and experiments using filtrates of old cultures may indicate the involvement of a cell-density dependant signalling molecule, acting as a negative repressor of the *pheN* gene. Such a mechanism may have parallels with the *lux* type of autoregulatory system (Bainton *et al*., 1992).

Such work demonstrates the emphasis that has been placed on brown blotch disease of mushrooms and the benefits of such research to our understanding of bacterial pathogenicity and ecology. The *P. tolaasii* system remains the most widely studied bacterial disease of *A. bisporus*. However, the potential economic benefits of a study of the rarer, but none-the-less destructive bacterial diseases has ensured constant vigilance and renewed research interest.

Ginger blotch

First described by Wong *et al.* (1982), sporadic outbreaks of this disease have been noted since 1977. Symptoms are similar to brown blotch, except that the cap lesions are characteristically pale yellowish-red, darkening to a ginger colour. Bacteria isolated from lesions and reproducing symptoms were fluorescent pseudomonads similar to *P. tolaasii*, but were negative in the white line test, did not cause rapid pitting of mushroom tissue blocks and did not agglutinate sera raised against *P. tolaasii*. The 2 species could also be separated by conventional biochemical tests

and later, the ginger blotch organism was named *Pseudomonas gingeri following* confirmation of its separate taxonomic status. Phenotypic variation similar to *P. tolaasii* was also noticed in *P. gingeri* (Cutri *et al.,* 1984), similarly affecting pathogenicity.

Mummy disease

Although uncommon, mummy disease can have a severe impact, especially on farms employing the deep trough production method, since the crop in the affected bed is often completely lost in subsequent flushes (Fermor, 1986; Fletcher *et al.,* 1989). This contrasts with blotch affected beds which, with effective disease management, can continue in production. Affected mushrooms have a dry, shrunken appearance and may die before opening. Stipe bases become swollen, and the stipes tough and fibrous. Uneven elongation of the stipe may also give the cap a tilted appearance (Tucker & Routien, 1942). The disease is also characterised by rapid spread once established, but spread to different growing units (trays, shelves etc.) is uncommon.

The etiology of mummy disease has always been controversial and several authors have claimed to have reproduced symptoms with dissimilar isolates. However, the disease was found to be transmissible by transplanting infected *Agaricus* colonised casing or compost, but not by transplanting affected sporophores (Tucker & Routien, 1942; Oxley, 1984), suggesting that living mushroom mycelium was necessary for disease spread; possibly indicating viral etiology. Studies in the UK by Bawden & Gregory (1951) and Storey (1954) though, failed to detect virus contamination in diseased sporophores. Schisler *et al*. (1968) isolated a fluorescent pseudomonad, similar to *P. tolaasii*, which could cause mummy symptoms in up to 35% of inoculations. Bacteria re-isolated from these infections appeared to be identical to those applied and could themselves initiate mummy disease. Such successful inoculation was achieved by mixing a 24 h culture of the bacteria into

freshly spawned compost. It was proposed that the bacteria entered the fungal mycelium as a result of physical damage and then continued to proliferate intracellularly, which was an explanation for the lack of spread of the disease between growing trays etc.; spread then being achieved by fungal anastamoses. Intracellular bacteria, appearing walled and rod-shaped, were also noted in mummy-affected mushrooms by Van Zaayen & Wattereus (1974) and Oxley (1984). Colonised hyphae displayed many abnormal features, including disorganised membranes and degraded cell contents. Adjacent cells lacking bacteria were found to have unusual wall thickenings, as was the case in mycelial cords. In attempts to reproduce the results of Schisler *et al*. (1968), Van Zaayen (1981) isolated similar bacteria, classified in biovar V of *P. fluorescens*; but could not reproduce the rates of infection achieved by the previous author. A similar result was obtained with Schisler's isolates, and so it was proposed that these isolates had either lost their pathogenicity, or had been poorly stored and become contaminated. Van Zaayen's isolates, however, also appeared to rapidly lose pathogenicity *in vitro*. This phenomenon could be related to the phenotypic variation seen in *P. tolaasii* and other fluorescent pseudomonads. Later work also failed to reach a consensus on finding a single, reliable bacterial cause for mummy disease. Oxley (1984), was also unable to reproduce mummy symptoms using the original isolates of Schisler, and a large collection of fresh isolates. Betterley & Olson (1989), however, achieved an infection rate of 40% with *P. fluorescens* biovar V strains isolated from mummy mushrooms. Interestingly, Wuest & Zarkower (1991) implicated *Pseudomonas aeruginosa* as the causal agent, since this species could be isolated from very young affected sporophores, and could reproduce symptoms. It was theorised that *P. aeruginosa* infects the mycelium, but is lost when other bacteria secondarily colonise affected mushrooms, thereby accounting for the low rates of recovery of this organism from previous studies of this disease. It was also suggested that

mummy disease could be caused by more than one species of bacterium, and might also be associated with interactions with pseudomonads and uncultured spiroplasmas or mycoplasma-like organisms. Research on this interesting syndrome has indicated the need for careful interpretation of bacteriological data, and suggests that the disease warrants further investigation in order to clarify the confused etiology.

Drippy gill and related disorders

Caused by *Pseudomonas agarici*, drippy gill is characterised by the development of dark, watersoaked lesions on the mushroom gills, that then go on to exude a white bacterial ooze (Young, 1970). The disease is sporadic and appears to infect mushrooms before the caps open, thereby suggesting that infection occurs at an early stage in mushroom development (Fletcher *et al.,* 1989).

Rainey & Cole (1988) described an outbreak in New Zealand, of a new disease somewhat similar to drippy gill, caused by *P. agarici.* Affected mushrooms were severely malformed, with the gill region being characteristically constricted. Profuse bacterial ooze was also produced from cut stipes. The causative organism, designated PMS-PV29, was weakly fluorescent and could be differentiated biochemically from previously described mushroom pathogens, including *P. agarici*.

Recently, a new mushroom disease has been attributed to *P. agarici* (Geels *et al*., 1994), symptoms of which take the form of a brown discoloration of caps, lacking the sunken appearance of *P. tolaasii*-caused lesions. Symptoms were reproduced using both bacteria isolated from affected mushroom caps and the type strain of *P. agarici*, LMG 2112. *P. agarici* is known to readily produce catechol from benzoate (Palleroni, 1984), which can then act as a melanogenic substrate for mushroom tyrosinase (see later), or be oxidised by the bacterium. This may possibly be an explanation for the brown pigmentation seen in the disease.

Bacterial pit

This disease is widespread and may be relatively serious, although lightly affected mushrooms may still be marketable. Symptoms appear as small, dark and often slimy pits in the cap surface, usually in later flushes (Fletcher *et al.,* 1989). Mites and nematodes have been implicated in the disease, but the most likely bacterial cause is *Bacillus polymyxa*, which can initiate the disease on artificial inoculation (Lelliot & Stead, 1987).

Diseases of other Agaricus species

With the adoption by some growers of higher temperature-requiring mushroom species such as *Agaricus bitorquis*, novel diseases have emerged, caused by bacteria, which may be favoured by such conditions. However, higher temperatures and a temporary change to an alternative species of *Agaricus* may be a suitable method to eliminate persistent virus disease (Atkey *et al.,* 1991; Smith *et al*., 1993).

Outbreaks of a severe soft rotting disorder have been reported by several UK growers of *A. bitorquis*. The disease is characterised by rapidly progressing, deep, oozing lesions on the cap surface. Lincoln *et al*. (1991) isolated a non-fluorescent pseudomonad from rotting mushrooms, that could reliably reproduce symptoms, both on entire mushrooms *in vivo* and in tissue blocks. Allied to *Pseudomonas gladioli* on the basis of automated fatty acid analysis and nutritional tests, the bacterium was proposed as a new pathovar, *P. gladioli* pv *agaricicola* on the basis of pathogenicity when compared to plant derived strains.

The above disease may be identical to the cavity disease of *A. bitorquis* described by Gill & Cole (1992) and attributed to *Pseudomonas cepacia*. Symptoms of this disease, noticed in New Zealand, are very similar or identical to the soft rot seen on UK farms. Additionally, in the earlier report, the taxonomy of *P. gladioli* pv *agariciola* was described as being very close to *P. cepacia.* Since the taxonomic

characterisation of the pathogen in the former report was more thorough than the later study, it is likely that the two diseases are synonymous.

Bacterial diseases of non- Agaricus mushrooms

The earlier part of this review indicated the growing popularity and importance of exotics such as oyster mushrooms (*Pleurotus* spp.) and shiitake (*Lentinula edodes*). Bacteria capable of infecting *A. bisporus* also appear, to a certain extent, to be capable of causing disease in other mushrooms (reviewed by Fermor, 1986). *P. tolaasii* causes serious problems in *Pleurotus* production, producing blotching symptoms similar to that seen in *Agaricus* and *P. agarici* has been shown to cause yellow blotch of *Pleurotus ostreatus* (Bessette *et al*., 1985). In some cases production of *Pleurotus* has become uneconomic, a situation which may be exacerbated by the common practice of growing this mushroom on a small scale as a sideline on *Agaricus* farms, thereby increasing the chances of cross-contamination with potential pathogens.

Shiitake mushrooms also appear to be susceptible to a number of bacterial diseases. A *P. fluorescens* strain has been implicated as the cause of a stunting and browning disorder in *Lentinula edodes* (Nakai *et al*., 1982). Another disorder, producing malformed sporophores, has been attributed to a rickettsia-like bacterium (Nakai & Ushiyama, 1985). Experimental evidence indicates that strains of *P. tolaasii* are strongly pathogenic against *L. edodes*, causing browning and lysis of the inoculated tissues. However, other strains of *P. fluorescens* were susceptible to bacteriolysis by *L. edodes* hyphae (Tsuneda & Thorn, 1994).

The "normal" bacterial flora of cultivated Agaricus mushrooms and growing media

The composting process is totally dependent on the actions of bacteria and thermotolerant fungi (Fermor *et al*., 1985) which, via downstream conditioning

procedures, becomes selective and suitable for *A. bisporus* mycelial growth (Figure 1). During the composting process itself, and in later pasteurisation steps, the majority of organisms with pathogenic potential are eliminated (Fletcher *et al.,* 1989). Additionally, the *Agaricus* mycelium itself is capable of causing bacteriolysis (Fermor *et al*., 1991) and may derive important growth requirements from such activity. The total microbial biomass of fresh mushroom compost has been estimated to be 2% of the compost dry weight, and so, though not a significant source of carbon, may be an important and concentrated source of nitrogen, lipids, vitamins and other trace nutrients (Fermor & Wood, 1991). It is the process of casing that stimulates *A. bisporus* to begin the reproductive phase of its growth, and it is this period that has the greatest influence on the final microflora of the mushrooms themselves. It is now widely accepted that it is the bacterial component of the casing layer that provides a significant influence on the initiation of the fruiting process, as well as the chemical and physical nature of the casing ingredients. This developmental requirement of *A. bisporus* has been described as an external fruiting stimulus (EFS factor), and has been shown to be provided by a wide range of bacteria, actinomycetes and fungi, usually of soil origin (O'Donoghue-Maguire & Ryan, 1991). Such EFS-providing species have included *Streptomyces* spp., *Bacillus psilocybe*, *B. megaterium, Arthrobacter* spp. and *Pseudomonas putida*. The microorganism that has received particular attention in this area is *P. putida.* First described by Hayes *et al*. (1969) in this context, *P. putida* was later shown to exert a stimulatory effect on *A. bisporus* mycelium, both in conditions simulating normal mushroom culture and in agar plate tests (Rainey, 1991a). *P. putida* increased the rate of radial hyphal growth and also caused suppression of branching on an agar medium incorporating compost extract and malt, but did not cause development of mushroom primordia. The hyphal changes *in vitro* were suggested as a prelude to later production of primordia, which were produced in the presence of *P. putida* and

casing materials. The fruiting stimulus therefore is likely to be complex and multifactorial. O'Donoghue-Maguire & Ryan (1991) suggested that chitinolytic microorganisms may have a particular influence on the supply of EFS factor. Evidence for this was provided by amending casing soils with chitin, which lead to raised chitinolytic streptomycete populations and concomitant increases in casing soil chitinase levels. This resulted in attenuated strand and rhizomorph development of *A. bisporus*. A possible mechanism is that chitinases render the mycelium more permeable, allowing either enhanced uptake of an unidentified stimulus or enhanced release of an inhibitor of the reproductive growth phase.

A positive requirement for bacteria in the casing layer means that this cannot be sterilised as a means of disease or bacterial colonisation prevention. However, seeding of the casing layer with disease-suppressive and fruiting- stimulatory bacteria may be a viable option. This bifactorial approach should therefore be considered when screening for potential biocontrol agents.

The postharvest quality of mushrooms has been shown to correlate closely with total bacterial numbers colonising the fruit bodies. Doores *et al.* (1986) showed that total aerobic counts of whole mushrooms was between 0.2 and 1.6 x 10^7 cfu g^{-1} on harvest, and rose to almost 1 x 10^{11} cfu g^{-1} during a 10-day storage study. During cropping, the casing layer yielded total aerobic counts of between 1.7 and 2.9 x 10^8 cfu g^{-1}, whereas immediately prior to application to mushroom beds, bacterial counts were 2.8 x 10^5 cfu g^{-1}. The fast growth rate of mushrooms or differences in nutritional content was proposed as an explanation for the almost 2 log cycle difference between the counts for the casing during cropping and for fresh mushrooms. Another possibility, however, may be the method chosen to evaluate bacterial numbers on mushrooms, since these were sampled and bacteriologically processed as a whole, thereby contributing a dilution effect. No attempt was made to evaluate the possible differences between the mushroom surface flora and that

colonising the mushroom interior. Bacteria isolated from mushrooms were predominantly (54%) fluorescent pseudomonads, identified as *P. putida* and *P. fluorescens*. Non-fluorescent pseudomonads comprised 16.6% of isolates and 2% of pseudomonads displayed a highly mucoid phenotype. Ten percent of isolates were *Flavobacterium* spp., identified by their bright orange pigmentation. *Moraxella* and *Acinetobacter* comprised 14.2% of isolates, and the remaining 3.2% were small numbers of *Bacillus, Micrococcus* and *Staphylococcus* species or unidentified isolates. In contrast, the bacteria isolated from mushroom-colonised casing material revealed a predominance (41%) of non-fluorescent pseudomonads, with fluorescent types only appearing, albeit in small numbers, in later flushes. Flavobacteria comprised 19% of isolates, while the *Moraxella - Acinetobacter* group accounted for 16% of the population. Miscellaneous unidentified strains accounted for 15% of the casing bacteria. The low numbers of *P. putida* and related fluorescent pseudomonads in the casing in this study is surprising, given their supposed importance in basidiome initiation and high numbers on mushrooms. An explanation for this could be the efficiency of the chemotactic response to *Agaricus* exudates (Grewal & Rainey, 1991) and adherence of these bacteria to mushroom surfaces (Rainey, 1991b). Coupled to this, the work of Wong & Preece (1982) showed that *P. tolaasii* (a fluorescent pseudomonad) did not persist in great numbers in uncolonised casing, which is likely to be similar in *P. putida*. The absence of *Streptomyces* spp. is also surprising, but is probably due to the bacteriological media and incubation conditions used. The populations of fluorescent pseudomonads in casing soils reported by Doores *et al.* (1986) is roughly in agreement with the findings of Samson *et al.* (1986), who also differentiated more thoroughly between the biovars of *P. fluorescens* and *P. putida*. *P. fluorescens* biovar V was the most common biovar in casing soil, forming 17% of the total fluorescent pseudomonad population, followed by biovar I at 9% and biovar III at 5.5%. *P. putida* accounted

for 47% of fluorescent isolates, but interestingly, strains intermediate between *P. putida* and *P. fluorescens* accounted for 21.5% of the population. This taxonomic uncertainty was brought about by the results for gelatinolysis, which classically is used to separate the two species. Such intermediate strains were also discussed in the extensive surveys of *Pseudomonas* conducted by Stanier *et al.* (1966) and Palleroni & Doudoroff (1972).

In addition to the association of *P. fluorescens* with cultivated mushrooms, similar relationships have also been reported in *Cantharellus cibarius*, an ectomycorrhizal mushroom (Danell*, et al*., 1993). Here, it was found that the *P. fluorescens* component of the bacterial population in the mushrooms soil environment was 12%, rising to 78% in the sporophores. Electron microscopy revealed that bacteria were embedded in mucus and were colonising the interhyphal spaces. It was suggested that bacteria became incidentally incorporated in the fruit body during its development. This situation may also be the case with *Agaricus* mushrooms, where development of sporophores from mycelial aggregations often results in fragments of casing material becoming trapped in the basal interior of mushrooms.

The physiological response of A. bisporus to infection

The most obvious symptom of ISN is the browning of the internal stipe tissues. This browning is progressive and appears to follow the course of the infection so that, at the stage when internal stipe tissue collapse ensues, browning is severe. The browning response may also extend into the internal cap tissues abutting the stipe, suggesting either spread of the causative agent to these tissues or the transport of a browning inducing agent into the cap.

Browning in mushrooms is largely due to the activity of the copper containing monophenol mono-oxygenase metalloenzyme, tyrosinase [EC 1.14.18.1] (Mayer & Harel, 1979). This enzyme is widely distributed in nature, but the

Agaricus enzyme has been widely studied, probably because of the availability and ease of extraction of the source. Tyrosinase is implicated in the pathway that leads to the formation of the pigment melanin, which may function to scavenge free radicals within cells, as well as affording protection from ultraviolet damage (Hearing, 1987). Melanin may also function to reduce the impact of microbial colonisation of mushroom tissues, since it has been shown to inhibit mycolysis and, in particular, chitinase activity (Bull, 1970).

In the most well understood reaction, tyrosinase initially catalyses the hydroxylation of tyrosine to 3,4-dihydroxyphenylanaline (DOPA). DOPA then acts as its own cofactor for the further enzymatic catalysis via tyrosinase to DOPA-quinone, which then oxidises spontaneously to melanin (Mayer & Harel, 1979; Vamos-Vigyazo, 1981). It has been shown that the enzyme has separate active sites for each catalytic activity (Maddaluno & Faull, 1988) and, at least in the mammalian enzyme, may also contain a third site for DOPA binding (Hearing, 1987).

Production of tyrosinase in *A. bisporus* is developmentally regulated. Turner (1974) showed that tyrosinase activity in colonised composts during the vegetative growth phase was extremely low when compared to activities following casing, during the reproductive phase. This relationship was previously noted in other fungi, notably in *Neurospora crassa* (Hirsch, 1954; Horowitz *et al*., 1960) and in *Hypomyces solani* (Wilson, 1968). Tyrosinase has been shown to be implicated in spore melanogenesis (Hegnauer *et al*., 1985), but in other tissues may function to confer resistance to microorganisms by virtue of the toxicity of the oxidation products (Wood *et al.,* 1991). Activation studies of tyrosinase by SDS and protease (trypsin) have shown that a significant proportion of the enzyme exists in a latent state in mushrooms (Yamaguchi *et al*., 1970; Flurkey & Ingebrigtsen, 1989). Activities of both active and latent forms were shown to decrease during development from pins to mature mushrooms, but this may be due to concentration

effects in the unexpanded tissues. During post-harvest storage or senescence, tyrosinase browning is thought to be due mainly to the activities of endogenous proteases, acting to both activate latent enzyme and to release enzyme / substrate from cells (Burton *et al*., 1993).

Thapa & Jandaik (1989) have investigated tyrosinase and peroxidase activities in *Agaricus* fruit-bodies during infection by *Verticillium fungicola* (Table 2). It was found that peroxidase showed a clear increase due to pathogen attack at all mushroom growth stages. Tyrosinase activity, however, did not increase due to infection significantly except in mushrooms that had just opened. Mushrooms at the fully open or "flat" stage were not affected. This provides evidence for the hypothesis that browning due to infection is most probably due to the action of pre-formed tyrosinase on released substrate.

Table 2. Oxidase activities in healthy and *V. fungicola* infected mushrooms. Data expressed as enzyme activity in Units ml^{-1} of extract; mean of 3 determinations. Table adapted from Thapa & Jandaik (1989).

	Peroxidase		Tyrosinase	
Mushroom Growth Stage	Healthy	Diseased	Healthy	Diseased
Pin	1.95	2.42	13.36	13.73
Button	1.57	1.97	15.13	15.93
Just open	1.75	1.82	10.80	12.20
Flat	1.95	2.52	9.60	9.73

In ISN, tyrosinase browning could be an active reaction by the mushroom to microbial attack. More likely however, as discussed above, is a passive response; due either to an exposure of pre-formed enzyme to compartmentalised substrate resulting from cellular damage, or an activation of enzyme activity by microbial or host released substances.

Progress on the molecular genetics of tyrosinase production in *A. bisporus* has, so far, been limited to the immunoprecipitation by anti-tyrosinase antibodies of *in vitro* translated mRNA (Podila & Flurkey, 1986). Although currently unavailable, a genomic clone could provide the opportunity to produce tyrosinase-attenuated strains, that might be cosmetically resistant to disease, and have an extended shelf-life. Tyrosinases have been cloned from several prokaryotic sources including *Streptomyces antibioticus* (Katz et al., 1983; Bernan *et al*., 1985) and *Streptomyces glaucescens* (Huber *et al*., 1985) and showed 86.4% peptide sequence identity. The tyrosinase from *Streptomyces michiganensis* was shown to be inducible by copper; and it was proposed that an element upstream of the pro-tyrosinase open reading frame in a polycistronic mRNA was responsible for incorporation of copper in the mature metalloenzyme (Held & Kutzner, 1990). Such cloned genes have been applied as reporters in promoter probe vectors, in particular, in work with non-melanogenic *Streptomyces* sp. such as *Streptomyces coelicolor* (Paget *et al*., 1994).

Among the fungi, the molecular genetics of tyrosinase of *Neurospora crassa* is most well understood. Tyrosinase is only transcribed during starvation or sexual differentiation in this species (Kupper *et al.,* 1990), which appears to parallel the situation in *A. bisporus*. More recently, tyrosinase genes and genes for related copper containing polyphenol oxidases have been cloned and sequenced from several plant, vertebrate and mammalian sources and appear to show significant homology to each other, in particular, in the copper-binding domains (Morrison *et al*., 1994). Such sequence conservation enhances the prospects for the design of probes for gene cloning in other organisms including *A. bisporus*. Genetic lesions in the tyrosinase locus could then be produced *in vitro* and the effects on growth, yield and quality assessed. Successfully modified strains of this type could then offer considerable economic advantages over conventional strains which are susceptible to premature browning and spoilage. Strain improvement along these lines may also be partially accomplished by adoption, or inclusion in breeding programmes, of exotic *Agaricus* species. Burton *et al*. (1993) found that 2 tropical *A. bitorquis* strains; *AGC W20* and ATCC32675, both had significantly lower levels of tyrosinase and phenols than the commonly grown *A. bisporus* strain U3. Correlated with this, the tropical species were both found to have a lower rate of post-harvest discoloration, although were not as white as the *A. bisporus* strain initially.

Ultrastructural changes in mushrooms due to infection have been investigated by several workers. Cole & Skellerup, (1986) described breakdown of the interhyphal matrix and hyphal disorganisation in lesions caused by *P. tolaasii*. Additionally, wall thickening occurred at the cap surface and where bacterial numbers were below a critical level, this was postulated as being a barrier to further bacterial colonisation. The mechanism or molecular stimulus for this response is not currently known, but it appears also to occur in mummy disease (Oxley, 1984),

where localised thickenings were observed adjacent to attached bacteria. The response was also observed to extend to adjacent uncolonised hyphae.

Virulence determinants in bacterial diseases of mushrooms

A discussion on the virulence determinants of bacteria affecting mushrooms must first address the characteristics of the host; in particular, the nature of the *Agaricus* cell wall. Additionally, of particular relevance to internal stipe necrosis where symptoms are confined to the internal stipe cells, is the possibility of differentiation between cells of the mushroom stipe and the rest of the mycelium.

The cell walls of most basidiomycetous fungi are composed of microfibrils of chitin embedded in a matrix of 1,3 linked and 1,6 linked β-D-glucan (Bartnicki-Garcia, 1968), which may have variable degrees of covalent interlinking (Sietsma & Wessels, 1979). Garcia Mendoza *et al*. (1987) showed that β-1,6 linked glucan was more common in stipe cells than in vegetative hyphae, possibly inhibiting the close packing of the 1,3 linked glucan and thus allowing more extensive expansion in these tissues. Elongation of stipes in *A. bisporus* has been shown to be dependent on the synthesis of chitin, since inhibitors of chitin synthase, such as polyoxin D arrest development (Wood & Hammond, 1977; Craig *et al*., 1981). Mol & Wessels (1990) showed that the walls of elongating stipe cells are more susceptible to chitinase than other hyphae, possibly as a result of a greater degree of acetylation in the stipe cells. Such differences may also be a consequence of the different organisation of wall components at the microfibrillar level between stipe cells and other cells. Mol *et al*. (1990) found that chitin chains were oriented transversely in the stipe and not organised into randomly distributed microfibrils embedded in an amorphous glucan matrix, as is the case with substrate hyphae. The chitin of the stipe cells is thus more available, particularly on the cell surface, to the action of chitinase, as well as being enzymatically more susceptible.

Of the currently recognised bacterial mushroom pathogens, we have information on virulence solely in the *P. tolaasii* system. The major factor influencing virulence in this organism is believed to be production of a membrane disrupting toxin, tolaasin, as discussed above. Other factors must, however, contribute to the effectiveness of the pathogen. Foremost among these is taxis, the ability to adhere to host surfaces, and the ability to compete for a niche with competitive saprophytes. The evidence of Wong & Preece (1982) suggests that *P. tolaasii* cannot proliferate in casing soils, and requires living *A. bisporus* mycelium; meaning that the bacterium must be able to actively migrate towards its host. Grewal & Rainey (1991) examined the chemotactic response of *P. tolaasii* and the saprophyte *P. putida*. Both bacteria displayed a positive chemotactic response towards mushroom mycelial exudate and to casamino acids (Casein hydrolysate). The attractants in the mushroom exudate appeared to be small molecular weight (M_r < 2000) thermostable compounds, which were most likely to be amino acids. Sugars present in the extracts did not cause a chemotactic response. Further work is required in order to determine the exact nature of the attractant involved in this response.

Preece & Wong (1982) investigated the attachment of *P. tolaasii*, *P. gingeri* and a number of saprophytes to *A. bisporus* and found that pathogenic forms adhered in significantly greater numbers (Table 3). This result was later confirmed by Rainey (1991b) with respect to the differences between phenotypic variants of *P. tolaasii* and *P. putida*. Electron microscopy of the attachment process revealed that *P. tolaasii* permanently adhered via fibrillar material that was quite unlike flagella and fimbriae (Preece & Wong, 1982). Similar results were obtained by Rainey (1991b), who also postulated that initial, reversible attachment was most likely to be brought about by electrostatic interactions.

Table 3. Percentage detectable attachment of various bacterial isolates to discs of the intact outer surface of mushroom (*A. bisporus*) caps 30 min after artificial inoculation. (Taken from Preece & Wong, (1982)).

Bacterial Species	Percentage detectable attachment (mean ± s.d.)
P. tolaasii NCPPB 3148	73.8 ± 14.5
P. "gingeri" NCPPB 3146	65.0 ± 15.0
P. "reactans" NCPPB 3149	30.6 ± 14.7
P. putida NCIMB 9034	31.0 ± 16.5
P. marginalis NCPPB 667	18.9 ± 10.8
Escherichia coli	23.3 ± 18.7

The competitive ability of *P. tolaasii* against many other potentially mushroom colonising bacteria, is possibly, besides a strong chemotactic response and adherence, to involve the wide spectrum of activity of tolaasin. As discussed earlier, this toxin can cause bacteriolysis of Gram-positive bacteria, although has little effect on Gram-negative cells. Evidence from biocontrol studies suggests that many other fluorescent pseudomonads have a similar competitive ability to *P. tolaasii* in the colonisation of *A. bisporus*. It is therefore probable that it is the host damage, brought about by toxin production, that allows *P. tolaasii* to proliferate in fresh lesions at the expense of saprophytes.

The extensive soft rotting seen in *A. bitorquis*, although not reported in the literature, is most probably due to massive production of mycolytic enzymes by *P. cepacia*. Soft-rotting of fruits and vegetables is thought to be promoted by production of pectolytic enzymes by pathogenic bacteria (Lelliot & Stead, 1987), and this is probably mirrored in this mushroom disease. Mycolytic enzymes are produced by a number of bacteria, and include the wall specific β-glucanases and chitinases (Peberdy, 1985). Chitinases may have particular importance to internal stipe necrosis pathogenesis due to the particular susceptibility of the stipe hyphae to this enzyme (Mol & Wessels, 1990; Mol *et al*., 1990). Perhaps most thoroughly

explored in biocontrol terms, chitinases have been shown to be important in a number of fungus / bacterial antagonistic interactions. Crude bacterial chitinase extracts derived from *Serratia marcescens* caused swelling and eventual lysis of hyphal tips of *Rhizoctonia solani* (Ordentlich *et al*., 1988), and a chitinolytic *Arthrobacter* sp. was shown to colonise and cause lysis of *Fusarium roseum* (Morrisey *et al*., 1976). Many alternative examples exist in this widely studied area.

Objectives of this research

The primary aim of this research was to confirm and accurately identify the causal agent of internal stipe necrosis of *Agaricus bisporus*, which previous evidence indicated was likely to be bacterial. This confirmation must be brought about by the application and proof of Koch's postulates, which was to be aided by the use of molecular methods of strain tracking and discrimination. During the course of this study, it was found that the causal bacterium was dissimilar to previously described mushroom pathogens, and so it was necessary to rigorously investigate the taxonomy of the organism. Additionally, this organism was shown to produce a chitinolytic enzyme, possibly related to its pathogenicity. Attempts were therefore made to characterise and clone this enzyme, as a prelude to confirmation of this characteristic as a virulence factor.

CHAPTER 2
Bacteriological analysis of internal stipe necrosis affected Mushrooms

INTRODUCTION

As discussed in Chapter 1, mushrooms have their own characteristic bacterial microflora, dominated largely by saprophytic fluorescent pseudomonads. Although of different bacterial composition, this situation has parallels with the microflora the exposed parts of plants and animals. In these cases, the bacteria that constitute the microflora may be totally benign, or be potentially pathogenic. The diseased state, in many systems, only being brought about by other factors that may either damage the host or upset the balance between potential pathogens and saprophytes, so that a pathogenic infection may begin. This is exemplified by brown blotch disease, where *Pseudomonas tolaasii* can exist in the steady state on mushroom caps alongside other fluorescent pseudomonads, but is encouraged to spread and cause serious lesions when the crop is overwatered, or exposed to changes in temperature and humidity causing condensation to form on the caps (Wong & Preece, 1982). A definite difference between the mushroom host and most plant and animal models is the essentially ephemeral nature of the sporophores. Bacteria are closely implicated with senescence or post-harvest deterioration (Beelman *et al.,* 1987; Guthrie & Beelman, 1987), meaning that microbiological investigations are essentially a race against time, in order to determine the true cause of a disorder, against a background of saprophytes. The rapid development and ephemeral nature of basidiomycete fruiting bodies provides a means of escape from certain diseases or microbial colonisation, thereby allowing the fungus to produce spores and complete its life cycle.

The 2-phase mode of development between mycelium and sporophore may involve different susceptibilities to bacterial infection. Bacterial colonisation of the mycelium may be manifested by various cropping disorders, but this is an area that is not well understood. Most evidence points to the mycelium having a fairly high resistance to bacterial invasion, and may itself have bacteriolytic properties (Fermor *et al*., 1991). The causative agent of mummy disease, however, is thought to be intracellularly associated with both vegetative mycelium and reproductive structures, since mycelium from affected crops is infectious (Oxley, 1988). More information on mycelial disorders is available regarding problems due to virus infection and also weed mould outbreaks in composts due to rapidly growing fungi such as *Penicillium* spp. and *Trichoderma* spp. (Fletcher, 1989).

The aims of the work described in this Chapter were to conduct a rigorous bacteriological investigation of mushrooms showing internal stipe necrosis symptoms, collected over a sufficiently long period to allow distinction between the true bacterial cause of the disease (if any) and colonising saprophytes. This is important, in that a single outbreak may not be representative of all cases of the disease. Since previous data indicated that the microflora of necrotic tissues was likely to be rich, selective and differential culture techniques were designed, in order to avoid underrepresentation of slower growing or scarcer bacteria. Following primary isolation, the bacteria are then identified using classical biochemical and nutritional profiling, or commercial rapid identification kits. Such techniques are aimed towards satisfying the first of Koch's postulates, that is, to demonstrate that a particular bacterial species or sub-species is *consistently* associated with the disease. This data may then be used to rationally select bacteria for further infectivity trials.

MATERIALS AND METHODS

Diseased mushroom collection

Mushrooms affected by ISN were regularly collected and analysed between April 1992 and September 1993. Samples were sourced from three UK farms: Middlebrook Mushrooms Ltd. Gateforth Farm, Selby, N. Yorks.; Thorpe Farm Mushrooms, Gateforth Park, Selby, N. Yorks; and Country Kitchen Foods (Middlebrook mushrooms), Langford, Avon. These samples were kept at 4˚C on receipt and examined bacteriologically within 36 hours of harvest.

Specimen preparation and primary isolation of bacteria

Preliminary investigations revealed that most stipe necrosis affected mushrooms were heavily colonised by fluorescent *Pseudomonas* sp. Further analyses, therefore, were designed to increase the differentiation of specific pseudomonads and to select for non-pseudomonad bacteria.

For quantitative bacteriological analysis, mushrooms were aseptically dissected, and symptomatic tissue collected, weighed (fresh weight) and placed in a sterile plastic universal bottle. To this was added maximum recovery diluent (MRD; Oxoid) measured to produce an initial ten-fold dilution. The tissue was then homogenised in a Silverson blender (with sterilised homogeniser blade assembly) for approximately 20 seconds, and the slurry serially diluted in MRD. These samples were then plated on selective and differential media and incubated as follows:

1. Luisetti agar (Luisetti *et al.,* 1972), incubated aerobically at 28˚C.
2. MacConkey agar (Oxoid) (MacConkey, 1909), incubated aerobically at 28˚C
3. LB agar (Miller, 1972), incubated anaerobically at 28˚C in a Merck anaerobic jar with anaerobic gas pack.

The Luisetti agar was prepared (l^{-1}) by combining 10 g sucrose, 10 g casamino acids (Difco), 30 g gelatin and 15 g agar (Difco) in 980 ml distilled water and autoclaving at 121˚C for 15 min. On cooling to 50˚C, 1.0 g K_2HPO_4 and 1.0 g

$MgSO_4.7H_2O$ were added, each as 10 ml sterile solutions and plates poured. Following incubation, bacteria were enumerated, and unique colonial types subcultured to LB prior to storage at 4˚C pending further characterisation of bacterial types. Colonies producing yellow / green pigments on Luisetti agar were categorised as presumptive *Pseudomonas fluorescens*, and further differentiated by observation of fluorescence colours with a hand-held UV lamp. Colonies were further differentiated by observation of a raised and mucoid colonial morphology. Such isolates were termed levan producing. Colonies growing anaerobically were subcultured onto MacConkey agar plates incubated as above, and categorised as either lactose fermenters or non-lactose fermenters. No strict anaerobes were isolated from mushrooms in this study, but would have been identified by their failure to grow following subculture and aerobic incubation. Isolates were examined microscopically following Gram's staining and screened for production of cytochrome C oxidase by blotting a representative colony with dried filter paper previously soaked in a solution of 0.1% (w/v) tetramethyl-*p*-phenylenediamine and 0.1% (w/v) ascorbic acid.

Preliminary comparative experiments using King's B medium (King *et al.*, 1954) and Luisetti medium for enhancement of fluorescent pigment production by *P. fluorescens* strains demonstrated the superiority of the latter medium. As well as proving more effective in stimulating pigment production, Luisetti agar allowed the direct identification of levan producing pseudomonads.

Preliminary identification of fermentative bacteria

Fermentative bacteria were preliminarily identified using API 20E strips (BioMérieux) set up according to manufacturer's instructions, but incubated at 28˚C for 48 h. Biochemical profiles were then interpreted using the API analytical profile index (Anon, 1989).

Identification of presumptive Pseudomonas species and biovars

Tests for carbon source assimilation were carried out following a modification of the methods of Stanier *et al*., (1966) and Palleroni & Doudoroff, (1972). Carbon sources were incorporated into agar plates using the minimal medium of Ayers *et al*. (1919), comprising (l^{-1}): $NH_4H_2PO_4$ 1.0 g; KCl 0.2 g; $MgSO_4.7H_2O$ 0.2 g; Bacto agar (Difco) 15 g; Bromocresol purple 0.02 g. Sugars were incorporated at 0.5% (w/v) and other carbohydrates at 0.1% (w/v). Bacteria were applied to plates using a 16-point replicator from master plate stocks kept on LB agar. Data was analysed using the probability matrix of Gennari & Dragotto (Table 1; 1992), programmed into a spreadsheet (Microsoft Excel), so that the closest match to the test profile could be calculated.

Miscellaneous characterisation tests:

Test for gelatinase

Production of gelatinase is, along with trehalose utilisation, one of the classical tests for the separation of *Pseudomonas fluorescens* and *Pseudomonas putida* (Stanier *et al.,* 1966; Palleroni & Doudoroff, 1972; Palleroni, 1984). *P. fluorescens*, being usually positive for both traits, and *P. putida* usually negative. A test was therefore developed that was both faster, and more sensitive than the usual gelatin liquefaction test. Gelatin (0.1% [w/v]) was added to LB agar and autoclaved at 121˚C for 15 min. Bacteria were applied as before to the solid medium in Petri dishes and incubated aerobically for 72 h at 28˚C. Plates were then flooded with a 0.01% (w/v) solution of Coomassie brilliant blue in 40% (v/v) methanol, 10% (v/v) acetic acid and left at room temperature for 15 min. Plates were then washed in distilled water and set aside

for 15 min, after which clearing zones around colonies representing gelatin degradation were observed.

Table 1. Probability matrix of Gennari & Dragotto (1992) for identification of oxidase-positive fluorescent *Pseudomonas*.

Pseudomonas species	P	Lev	Den	LAr	DXy	Tre	Muc	Eri	mIn	Mes	Eth	Ant	His	Tri	Qui
fluorescens V-1	0.01	0.01	0.01	0.99	0.76	0.84	0.99	0.01	0.01	0.01	0.01	0.01	0.11	0.97	0.97
fluorescens V-2	0.01	0.01	0.01	0.8	0.01	0.01	0.99	0.01	0.01	0.01	0.01	0.01	0.99	0.99	0.99
fluorescens V-4	0.01	0.01	0.01	0.01	0.01	0.99	0.01	0.01	0.99	0.33	0.99	0.99	0.99	0.33	0.99
fluorescens V-5	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.99	0.01	0.1	0.01	0.99
fluorescens V-6	0.01	0.01	0.01	0.99	0.94	0.99	0.99	0.88	0.99	0.99	0.69	0.99	0.12	0.94	0.99
fluorescens V-7	0.01	0.01	0.01	0.01	0.99	0.99	0.01	0.01	0.01	0.01	0.67	0.01	0.01	0.01	0.99
fluorescens I-1	0.01	0.9	0.01	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.2	0.8	0.5	0.2	0.8
fluorescens I-2	0.01	0.9	0.01	0.8	0.2	0.8	0.8	0.2	0.2	0.2	0.2	0.2	0.8	0.8	0.8
fluorescens II-1	0.01	0.9	0.9	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.5	0.2	0.8	0.8
fluorescens II-2	0.01	0.9	0.9	0.8	0.2	0.8	0.8	0.2	0.8	0.8	0.8	0.2	0.2	0.8	0.8
fluorescens II-3	0.01	0.9	0.9	0.8	0.8	0.8	0.8	0.2	0.8	0.2	0.5	0.8	0.2	0.8	0.8
fluorescens III-1	0.01	0.01	0.9	0.2	0.2	0.8	0.8	0.8	0.8	0.5	0.8	0.8	0.2	0.2	0.8
fluorescens III-2	0.01	0.01	0.9	0.2	0.2	0.8	0.2	0.8	0.8	0.8	0.2	0.8	0.2	0.2	0.8
fluorescens IV-1	0.5	0.9	0.9	0.8	0.5	0.8	0.8	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.8
fluorescens IV-2	0.5	0.9	0.9	0.8	0.8	0.8	0.8	0.2	0.8	0.2	0.8	0.2	0.8	0.2	0.8
fluorescens K	0.01	0.01	0.9	0.8	0.2	0.8	0.8	0.2	0.2	0.8	0.2	0.2	0.8	0.8	0.8
fluorescens L	0.01	0.01	0.9	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.8	0.2	0.8	0.8	0.8
chloroaphis	0.9	0.9	0.9	0.2	0.2	0.8	0.8	0.5	0.8	0.8	0.8	0.8	0.8	0.2	0.8
aureofaciens	0.9	0.9	0.01	0.8	0.2	0.8	0.8	0.2	0.8	0.8	0.2	0.8	0.8	0.2	0.8
lunadensis	0.01	0.01	0.01	0.99	0.01	0.6	0.01	0.02	0.9	0.01	0.05	0.01	0.01	0.01	0.01
putida A	0.01	0.01	0.01	0.22	0.09	0.01	0.99	0.01	0.01	0.12	0.91	0.03	0.99	0.99	0.99
putida B	0.01	0.01	0.01	0.94	0.82	0.06	0.99	0.01	0.06	0.01	0.76	0.99	0.82	0.82	0.99
aeruginosa	0.86	0.01	0.99	0.1	0.1	0.1	0.1	0.1	0.1	0.9	0.9	0.9	0.9	0.1	0.5

P, Phenazine pigment; Lev, levan production; Den, denitrification. Assimilation of: LAr, L-arabinose; DXy, D-xylose; Tre, trehalose; Muc, mucic acid; Eri, erithritol; mIn, myo-inositol; Eth, ethanol; Ant, anthranilic acid; His, histamine; Tri, trigonneline; Qui, quinic acid. Numbers refer to the percentage (/100) of isolates in a particular taxon, scoring positive for that character. Total scores were calculated on the basis of these *P* values if the character was positive and 1-*P* if negative. Results were then normalised, and relative identification percentages calculated (% id, Anon, 1989).

White line in agar test for P. tolaasii

Wild-type cultures of *P. tolaasii* and *Pseudomonas "reactans"* were obtained by plating out material derived from a mildly blotched mushroom cap on Luisetti agar. Pairs of colonies displaying the white line precipitation reaction (Wong & Preece, 1979) were isolated, and the identity of *P. tolaasii* confirmed by a mushroom tissue block rapid pitting test. These two cultures were then used in streak tests on Luisetti agar, to screen ISN isolates either for identity as *P. tolaasii*, or ability to instigate a white line reaction with the confirmed *P. tolaasii* isolate.

Production of chitinase

CM-chitin-RBV (5 mg ml^{-1}; for preparation, see Chapter 5) was added to molten 2x LB agar at 50% (v/v) and plates poured. This medium was then used to screen for chitinase production by isolates, using the multi-point inoculator as previously described. Plates were incubated for 72 h at 28°C, after which, a positive reaction was indicated by the development of clearing zones in the coloured medium.

Production of lipase (Tween 80 esterase)

Tween 80 was autoclaved at 121°C for 15 min and added at 1% (v/v) to a separately autoclaved basal medium comprising (l^{-1}) 10 g peptone, 5 g NaCl, 0.1 g $CaCl_2.2H_2O$, 15 g agar (Lelliot & Stead, 1987). Plates were poured and bacteria applied as above. A positive result was indicated by the development of white halos around lipolytic colonies.

Production of siderophores

A plate screening assay for the detection of siderophores was conducted in according to Schwyn & Neilands (1987), using a complex of chrome azurol S / iron (III) / hexadecyltrimethylammonium bromide as indicator of chelating activity. One litre of medium was prepared by first dissolving 60.5 mg chrome azurol S (Fluka) in 50 ml distilled water and adding 10 ml of a solution of 1mM $FeCl_3.6H_2O$ in 10mM HCl. While stirring, a solution of 72.9 mg hexadecyltrimethylammonium

bromide (Sigma) in 40 ml distilled water was added and the resulting solution autoclaved at 121˚C for 15 min. A basal medium was prepared by dissolving 1 g $MgSO_4.7H_2O$ and 30.24 g PIPES (1,4-piperazinediethanesulfonic acid) in 750 ml distilled water and adding approximately 12 ml 50% (w/v) NaOH until the pH reached 6.8. Agar (15 g: Difco) was then added and the mixture autoclaved at 121˚C for 15 min. On cooling to 50˚C, 30 ml each of 10% (w/v) casamino acids (Difco) and 10% (w/v) sucrose were added as sterile solutions to the basal medium. The blue dye complex solution was then slowly added with gentle mixing and plates poured. Bacteria were applied as described previously and plates incubated for 72 h at 28˚C. A positive result was indicated by the development of orange zones around siderophore producing colonies. *E. coli* strain DH5α was used as negative control.

Haemolysis

Warmed defibrinated horse red blood cells (Oxoid) at 5% (v/v) was added to molten nutrient agar (Oxoid) at 45˚C. Following gentle mixing, plates were poured and bacteria applied using the multipoint technique. Plates were then incubated for 72 h at 28˚C. A positive result was indicated by the development of clearing zones around colonies representing lysis of the red blood cells. A wild-type *P. tolaasii* strain was used as positive control.

Histochemical staining of tissue blots for mushroom tyrosinase

Methods were adapted from Spruce *et al.* (1987) and Moore *et al.* (1988), in order to demonstrate the presence in diseased specimens of the major browning enzyme in mushrooms, tyrosinase. Mushroom sections were pressed lightly onto the surface of a nylon hybridisation membrane (Hybond N, Amersham) for 30 s and the blots rinsed once in 100 mM sodium phosphate buffer, pH 6.0. Filters were then developed by soaking for up to 1 min in a 0.5 mM solution of L-DOPA (Sigma),

until colour development was optimal. Developed blots were then rinsed in distilled water and allowed to dry at room temperature in the dark.

RESULTS

The number of bacteria isolated from stipe necrosis affected mushrooms was usually in the range of 10^8 - 10^{10} cfu g^{-1} (fresh weight) whereas normal, healthy internal stipe tissues were found to have a total bacterial population of 0-10^3 cfu g^{-1}. Bacteria could be divided into two categories: fluorescent pseudomonads and a tight cluster of coliform bacteria (lactose fermenting *Enterobacteriaceae*). Anaerobic conditions for 72 h effectively suppressed growth of the pseudomonads, allowing isolation and subculture of fermentative bacteria. On aerobic plates, the faster growing pseudomonads overgrew the coliforms and prevented their detection. The pseudomonads were identified and sub-divided according to the probability matrix of Gennari & Dragotto (1992), and were found to belong largely to biovars K, V-6 and IV-2 of the *P. fluorescens* complex (Table 2). Variability within these groups was significant, and the isolates probably form a relatively diverse assemblage. In contrast, the enteric bacteria form a homogenous group. Most of these bacteria are characterised by a chitinolytic and lipolytic (Tween 80 esterase) phenotype and probably constitute a distinct species. At least 90% of the chitinase-and lipase-positive enteric group, when analysed using API 20E, gave the profile code 1205101, which was named as *Ewingella americana* using the third edition of the API analytical profile index (20E), with a 99.1% identification probability (Anon, 1989). Several additional enteric strains were sporadically isolated, but these were not reliably identified using the API system.

Table 2. Frequency of isolation of bacterial species and sub-species from stipe necrosis affected mushrooms analysed between 1992 and 1993 (Total 180 isolates; 70 individual samples).

Species	Frequency of isolation (%)
E. americana (API 1205101)	93
P. fluorescens biovar K	53
P. fluorescens biovar IV-2	40
P. fluorescens biovar V-6	21
P. putida B	11
Unidentified enterics	9
P. fluorescens biovar III-1	9
P. fluorescens biovar III-2	4
P. fluorescens biovar II-1	4
P. paucimobilis	1
P. fluorescens biovar III-3	1

Most samples were colonised by a mixed bacterial population. A fluorescent pseudomonad was present in most cases, at a count of between 1.4×10^7 - 7.2×10^{10} cfu g^{-1}, along with lower counts of enteric bacteria ranging from 3.6×10^3 - 6.7×10^6 cfu g^{-1}. Often, 2 fluorescent pseudomonads were co-dominant and could be clearly differentiated on Luisetti agar on the basis of colony morphology, levan production or differences in fluorescence colour when examined under UV light. These differences correlated well with the biochemical identification data. No single group of *P. fluorescens* strains was consistently isolated over the study period however. Colonisation by particular biovars appeared to be sporadic and related either to the source of the samples, or different disease outbreaks. Strains identified as *E. americana,* however, were consistently isolated over the study period, from all farm sources and from all outbreaks.

Table 3. Distribution of potentially significant characteristics among the taxonomic groupings of bacteria collected from ISN affected mushrooms. (see text for methodologies).

Species	No.	Chi	Lip	Hae	Gel	Mil	Sid	Win
E. americana (API1205101)	66	66	66	0	0	0	66	0
P. fluorescens biovar K	37	1	3	2	3	7	37	0
P. fluorescens biovar IV-2	28	0	28	0	8	15	28	0
P. fluorescens biovar V-6	15	0	1	5	7	9	15	7
P. putida B	7	0	0	0	0	0	7	1
P. fluorescens biovar III-1	6	0	4	4	5	5	6	4
P. fluorescens biovar II-1	3	0	0	0	0	2	3	1
P. fluorescens biovar III-2	2	2	1	0	0	0	2	0
Pseudomonas paucimobilis	1	0	0	0	1	1	1	0
P. fluorescens biovar III-3	1	0	0	0	0	0	1	0

No., number of isolates; Chi, Chitinase; Lip, Tween 80 hydrolysis; Gel, Gelatin degradation; Mil, Casein hydrolysis (Skimmed milk); Sid, siderophore production; Win, White line in agar test indicator activity (*P. "reactans"*). Numbers refer to isolates positive in a particular character.

The variation within the major *P. fluorescens* clusters, although still allowing probable identification to the biovar level, was significant (Table 3). The results for gelatin degradation were variable and no *P. fluorescens* group reliably expressed this character. Casein hydrolysis was used as an alternative test for protease production. Several more strains were detected as being positive by this test than identified by the gelatin test, although these were still in a minority. This may have provided evidence for classifying other strains as *P. putida*, but the majority of these were trehalose-positive, which is a *P. fluorescens* trait. This probably reflects the difficulties in unambiguously classifying these strains, as found by Stanier *et al.* (1966), who admitted that many strains are refractory to classification and probably form a continuum with *P. putida*. This same conclusion was made by Barrett *et al.* (1986), who placed certain *P. fluorescens* biovar V strains close to *P. putida*. Interestingly, 7 strains identified as *P. fluorescens* biovar V-6, 4 strains of *P. fluorescens* biovar III-1 and 1 strain each of *P. fluorescens* biovar II-1

and *P. putida* gave a positive result as indicators in the white-line-in-agar test (Wong & Preece, 1979), which has been reported as being specific for the so-called *P. "reactans"*. If the identifications made here are reliable, then this points to the possibility that several mushroom associated *P. fluorescens* types are capable of producing the white line reacting principle. The identification of *P. fluorescens* biovar IV correlated perfectly with the production of a blue, non-diffusible pigment by these strains after extended incubation on Luisetti agar, which is diagnostic for this subdivision (Palleroni, 1984), and helps to counteract the uncertainty regarding the gelatin hydrolysis test. Similarly, easily scored marker characters such as levan production also correlated with the probabilistic identifications.

Among the *P. fluorescens* groups, there was little consistent expression of a single character which may be regarded as related to virulence or host damage. An exception was lipase production by *P. fluorescens* biovar IV-2, which was also positive for all *E. americana* isolates. All the pseudomonads and all *E. americana* strains displayed siderophore activity. The negative control and several unidentified enteric bacteria produced no zone. This result is probably related to iron chelating pyoverdin or pyocyanin pigment production by the pseudomonads and to unidentified siderophore production by *E. americana*. Chitinase production was rare among the pseudomonads, but was positive in all *E. americana* isolates. Horse red blood cell haemolysis was positive in only a few strains, in particular, in 33% of the *P. fluorescens* biovar V-6 isolates.

Histochemical staining for mushroom tyrosinase failed to detect a concentration of the enzyme in ISN affected regions of the stipe (Figure 1). However, concentrations of tyrosinase could be visualised in the pilei pellus (cap epidermis) and gill regions, which is consistent with the findings of Moore *et al.* (1988).

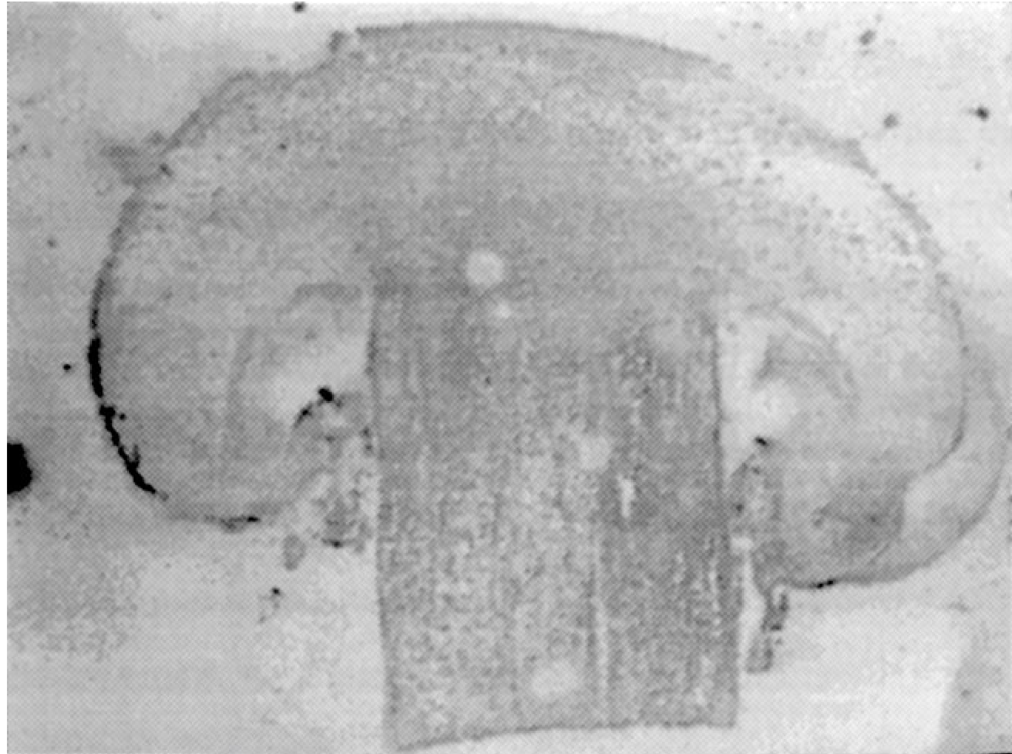


Figure 1. L-DOPA treated blot of a mushroom longitudinal section, which displayed typical internal stipe necrosis symptoms. (Image magnified x1.6).

DISCUSSION

Numbers of bacteria isolated from ISN affected mushrooms, being in the range 10^8 - 10^{10} cfu g^{-1} (fresh weight), are significantly higher than the figure for fresh mushrooms as found by Doores *et al.* (1986), which was in the range of 0.21 to 1.6 x 10^7 cfu g^{-1} (fresh weight). Additionally, the control figure found in this study was up to 10^3 cfu g^{-1} (fresh weight), which is substantially below the numbers reported in previous studies. This result was probably a consequence of the sampling method adopted, in that internal tissues were aseptically sampled, thereby avoiding gross surface bacterial contamination. Doores and co-workers (Doores *et al.*, 1986) processed whole mushrooms, thereby skewing the results; surface counts probably being substantially in excess of the reported figure, and internal counts substantially

lower. The work of Peng (1986) has indicated that non-pathogenic bacteria cannot penetrate deeply into mushrooms. Even *P. tolaasii*, in sub-critical numbers (Wong & Preece, 1982), or in typical blotch lesions (Cole & Skellerup, 1986), cannot penetrate into the cap past the barrier of the cap epidermis, or past the collapse of surface hyphae due to infection. Possible explanations for this resistance could be the increased levels of inhibitory phenols in these regions (Burton *et al*., 1993) and the close packing of the hyphae. Chance incorporation of saprophytic bacteria during sporophore initiation probably accounts for the comparatively small numbers that were found in internal stipe tissues.

The finding of *P. fluorescens* in association with internal stipe necrosis affected mushrooms is in agreement with the data of Richardson (1993), but in this previous report, no attempt was made to quantify or fully identify the strains collected. The sample size was also extremely small (5) and collected from a single source / time. This contrasts with the present study where 70 separate mushroom samples were collected from 3 separate farms during a period of 18 months. Isolates have been fully identified to beyond the biovar level and significant non-pseudomonad bacteria detected in the majority of samples. The first of Koch's postulates states (see Chapter 3) that a pathogen must be consistently associated with the disease under investigation. The *E. americana* isolates discovered in ISN affected mushrooms appear to satisfy this criterion, although are isolated in lower numbers than the *P. fluorescens* isolates. This may be explained by the relatively fast growth rate of *P. fluorescens* at lower temperatures (Gennari & Dragotto, 1992), as encountered during the mushroom cropping phase (see Chapter 1). As a species, *P. fluorescens* was also consistently isolated from diseased mushrooms. However, when examined at a finer taxonomic level, the isolates were found to be relatively diverse and assignable to different valid subdivisions, or biovars and their subgroups. The taxonomy of *P. fluorescens* and the phytopathogenic fluorescent

pseudomonads have been discussed in this context by Dye *et al*. (1975) who described its subdivisions as having "phytopathogenic individuality”. This means that *P. fluorescens*, at a functional level, cannot be regarded as a single species. Evidence supporting this idea has been supplied subsequently in several studies, which describe the ecological isolation of the biovars and biovar divisions within the *P. fluorescens* complex. Thus, *P. fluorescens* biovar V-1 was the most commonly isolated from soil and food (Barrett *et al.*,1986), biovar I-1, the most common from milk and cheese, V-6, most commonly found in spoiled fish and water and K, most commonly found in soil (Gennari & Dragotto, 1992). The only detailed study of this nature concerned with mushroom microflora was made by Samson *et al.* (1986), who identified biovar V as forming 20% of the fluorescent pseudomonad population of compost and 17% of the casing population (Table 4). No subdivision of biovar V was made, nor biovar K recognised, although these two groups are related (Champion *et al.,* 1980; Barrett *et al.,* 1986).

Table 4. Distribution of the species and biovars of fluorescent *Pseudomonas* isolated from composts and casing soils. (Adapted from Samson *et al*., 1987).

Pseudomonas species and biovars	Proportion (%)	
	Compost	Casing
P. fluorescens biovar I	6	9
P. fluorescens biovar II	2	-
P. fluorescens biovar III	5	5.5
P. fluorescens biovar V	20	17
Intermediate *fluorescens-putida* A	3	8
Intermediate *fluorescens-putida* B	8	13.5
P. putida	56	47

Such data would indicate that with the frequency of isolation of *P. fluorescens* and *P. putida* from composts and casing soils, a much wider prevalence for internal stipe necrosis might be expected and additionally, the disease might have been identified

much earlier. Rather, this disease is somewhat unusual and has only been recognised since 1991. Reasons for this recent emergence are possibly related to changes of practice within the mushroom industry and to factors such as sources of supply of peat and other materials. There is evidence that peat used for casing materials is being more deeply extracted as this resource becomes rarer and more costly. This may introduce formally unfamiliar bacterial species, such as *E. americana* into mushroom growing; which, being facultatively anaerobic, may be adapted to deep peat and anaerobic soils. Additionally, the practice of mechanically ruffling the mushroom beds following casing, in order to produce a more uniform crop amenable to machine harvest might increase the chances of potentially pathogenic bacteria becoming associated with damaged mycelium (J.L. Burden, personal communication).

Perhaps of greater significance than taxonomic assignment is the lack of any linking character, besides siderophore activity, among the *P. fluorescens* strains that possibly relate to pathogenicity. Siderophores may have little significance in this system, since in previous studies, alternative modes of antagonism have been shown to play a greater role (Oedjijono *et al*., 1993) and in addition, *A. bisporus* has been shown to possess its own siderophores and receptors (Engwilmot *et al*., 1992).

Chitinase is of possible significance to internal stipe necrosis, due to the unique vulnerability and availability of the chitin of the *Agaricus* stipe hyphae (Mol & Wessels, 1990; Mol *et al*., 1990). While rare among the *P. fluorescens* strains, this character was present in all *E. americana* strains. The lipolytic activity of these strains may also contribute to invasiveness.

Previous studies of bacterial diseases of mushrooms suggest that a single bacterial "species" is normally implicated in a particular disease. An exception is mummy disease, but as discussed in Chapter 1, the etiology of this syndrome remains controversial. The characteristics of mixed infection could present

complications in the interpretation of Koch's postulates. However, the specific nature of the disease, with it's clearly defined and limited symptoms, suggests that it is a true disease, and not a non-specific rot. Coupled to this, the diverse taxonomic nature of the pseudomonads encountered in specimens most probably implies simple, non-specific colonisation by these strains. The conclusion follows that *E. americana* probably initiates infection and that *P. fluorescens* isolates represent highly competitive secondary colonisers. On this evidence therefore, the group provisionally identified as *E. americana* was chosen for further investigation in Koch's postulate experiments.

The histochemical staining experiment for mushroom tyrosinase failed to detect any concentration of this enzyme in symptomatic tissues. The observed browning therefore is probably caused by the exposure of pre-formed enzyme to its similarly compartmentalised substrate due to hyphal damage by the colonising bacteria. Ultrastructural studies might confirm whether these bacteria are present intercellularly or extracellularly. A certain amount of cellular penetration and damage may be assumed however, since the disease is observed to progress to the stage where the browned tissue physically collapses. Electron microscopical investigations would be of assistance here, particularly when coupled with immunostaining procedures for the *in situ* identification of bacteria within necrotising areas. Whether the observable damage and symptoms are directly caused by the primary pathogen, secondary colonisers or a host response remains uncertain.

CHAPTER 3
The application of Koch's Postulates to internal stipe necrosis of *Agaricus bisporus*

INTRODUCTION

In the last quarter of the 19th century, Robert Koch firmly established the *germ theory of disease* by formulating a set of rules to justify the idea that a specific organism causes a specific disease. His criteria, now known as Koch's postulates state:

1. The organism should always be associated with diseased, but be absent in healthy, tissues.
2. The organism must be cultivated in pure culture away from the host.
3. When inoculated into a healthy host, the organism should initiate the characteristic disease symptoms.
4. The organism should be reisolated from the experimental host and shown to be identical to the applied organism.

These statements laid the foundation for subsequent studies in microbial pathology; and the principles must still be applied today in cases of diseases caused by microorganisms that can be cultivated in the laboratory. However, our knowledge of microbial variation and the application of modern methods of taxonomy, means that we must be assured that the applied strain being investigated is identical to strains recovered from deliberate infections, and that our conclusions are not based on mere assumption. Additionally, a pathogenic microorganism may regularly form

part of the normal microflora of a potential host, and the disease-causing potential of that microorganism only be realised by a conjunction of a number of biotic and abiotic factors that precipitate symptom development.

Molecular techniques in bacterial epidemiology

Differentiation and typing of bacteria, based on classical phenotypic characters is often limited by inherent variability and problems in verification (Allardet-Servent *et al.,* 1989). Also, there is a lack of available identification systems in many bacterial groups; particularly those not of medical importance. The ability to accurately 'fingerprint' strains is essential for the understanding of that strain's epidemiology (Jordens, 1991). Furthermore, an understanding of the true variability of bacteria within a set of isolates may be of significance if we consider the prevalence and establishment of a specific disease syndrome.

The resolution of typing strategies is a vital consideration. Classic biochemical and nutritional identification techniques (e.g. API systems, Bergey's manual etc.) may be adequate in the identification of a bacterial species, but the inherently probabilistic nature of the techniques make classification to the strain level difficult. Recently, rapid and sensitive systems have been developed, (e.g. BIOLOG, Jones *et al*., 1993), but strain characterisation is not claimed. Of the automated systems available, analysis of bacterial fatty acid composition has received attention and has been applied to the identification of plant pathogens (Stead *et al.,* 1992). In some cases, subdivisions have been defined within species (Mukwaya & Welch, 1989). Markers based on genome analysis are generally considered to be more sensitive indicators of the epidemiology of a particular strain. These must be chosen and applied with care; e.g., 16s rDNA sequencing is a powerful technique in the definition of higher-level taxonomy, but would be

cumbersome and insensitive in detecting the differences between closely related strains.

Molecular fingerprinting is an attractive alternative to genetic marking of strains destined to be released into the environment during Koch's postulate experiments. This is particularly important in the light of the restraints imposed on the release of genetically modified micro-organisms (Prosser, 1994). Genetic marking of strains, however, remains a powerful technique and is useful in many *in vivo* experiments, particularly in non-sterile systems. Of the many diverse reporter genes used to label bacteria, bioluminescence genes have recently attracted particular attention (Shaw *et al.,* 1992; Stewart & Williams, 1992; Beauchamp *et al.,* 1993). Bioluminescence is rare in non-marine bacteria (an exception is *Xenorhabdus luminescens*, a coloniser of nematodes and insect larvae; Schmidt *et al*., 1989), increasing the resolution of the technique in complex micro-communities. Constructs comprising the complete *lux* operons of *X. luminescens* and *Vibrio fischeri* are available (Stewart & Williams, 1992), enabling transformed bacteria to luminesce without external application of substrate. Modern instrumentation can detect extremely small numbers of luminescing cells (Grant *et al*., 1991), thereby expanding the application of the technique to the study of the development of bacterial microcolonies from individual cells.

Molecular typing methods can be divided into those that analyse the whole bacterial genome, those that analyse sequence variation within specific genes and those that detect and differentiate extrachromosomal markers. Plasmid typing is an example of the latter category and has been applied extensively in medical bacteriology (Noble & Rahman, 1986; Centron Garcia *et al*., 1989). Modifications involving restriction enzyme analysis of plasmids has increased the reliability and resolution of the technique (Hartstein *et al*., 1989). This method, however, is limited to those strains carrying plasmids, and a major criticism of it's applicability is the

possibility that these may be lost, gained or modified during environmental release or on prolonged culture. Methods which analyse the whole genome or specific genes are perhaps more stable and reliable techniques for monitoring bacteria. Most methods utilise restriction enzymes to fragment the genome, but differ in the techniques whereby patterns are visualised. In this case, it is the various restriction sites on the genomic DNA that serve as molecular markers resulting in detection of restriction fragment length polymorphism's or RFLP's. Sequence variation has been detected in a number of conserved genes and in some cases, have proved to be valuable epidemiological markers. Of these, the ribosomal genes and their intergenic spacers have received particular attention (Grimont & Grimont, 1986; Priest *et al*., 1994). Ribotyping utilises frequently cutting restriction enzymes to cleave ribosomal genes previously amplified by PCR, or cloned via reverse transcriptase. Highly resolving electrophoresis apparatus is then used to visualise the resulting fragments. A limitation of the technique is the comparative stability of ribosomal genes at the sequence level, meaning that the technique may not differentiate closely related strains.

RFLPs utilising the whole bacterial genome have perhaps been the most successful technique in epidemiological monitoring. Many techniques employ infrequently cutting restriction enzymes to fragment agarose-immobilised chromosomal DNA's (McClelland *et al.,* 1987). The macrorestriction patterns are then resolved using pulsed-field electrophoresis (Grothues & Tümmler, 1987; Allardet-Servent *et al.,* 1989; Prévost *et al.,* 1991; Holloway *et al.,* 1992; Buchrieser *et al*., 1993). Another approach is to fragment genomic DNA with common restriction enzymes and prepare Southern blots to detect probe-hybridisation patterns. The filters are then probed with suitable marker genes that are conserved among the range of strains being examined. A range of genes have been employed; e.g.. exotoxin A and pilin genes of *Pseudomonas aeruginosa* (Samadpour *et al.,*

1988; Speert *et al.,* 1989) and the elongation factor (EF-Tu) gene of *Mycoplasma* spp. (Yogev *et al*., 1988). The method has greater resolution if the gene is present in multiple copy and is highly conserved. Ribosomal genes fall into this category and have been widely used (Pitcher *et al.,* 1987; reviewed by Owen, 1989).

The source of the variations observed in RFLP analysis has been investigated in *Enterococcus faecalis* by Hall (1994), with the finding that DNA rearrangements are more commonly implicated, rather than point mutations in restriction sites. The genome of *Escherischia coli* also demonstrates similar large-scale rearrangement due to transposition by prophages, insertion sequences and conjugative mating with HFR strains (Stolzfus *et al*., 1988).

The above account of techniques used in bacterial strain analysis is by no means exhaustive. No mention has been made of the many emerging PCR based techniques such as randomly amplified polymorphic DNA (RAPD) etc. Each method should be chosen according to the purpose and group to which it is to be applied, and in the case of non-cultivatable microorganisms, PCR offers an excellent opportunity to develop typing schemes.

The aims of the work described in this chapter are to apply Koch's postulates to mushroom ISN and to develop a molecular fingerprint of the causative agent. As stated in Chapter 2, the enteric cluster of stipe necrosis derived bacteria, provisionally identified as *Ewingella americana*, were selected for trial since they appear to satisfy the first criterion, namely, to be consistently associated with the disease.

MATERIALS AND METHODS

Strains and vectors

The commercial mushroom strain, *A. bisporus* Somycel 512 was used in re-inoculation experiments with stipe necrosis isolates PI4, PI74, PI98, PI145 & PI180. Strain PI4 is a *P. fluorescens* biovar K strain, atypically producing an extracellular chitinase. Other bacterial strains are all enteric cluster isolates recovered from separate outbreaks.

Escherichia coli Strains DH5α (BRL) Phenotype: F^-, φ80d*lac*ZΔM15, Δ(*lac*ZYA-*arg*F), U169, *deo*R, *rec*A1, *end*A1, *hsd*R17(r_k-, m_k+), *sup*E44, λ^-*thi*-1, *gyr*A96, *rel*A1 and XL1-Blue MRF′ (Stratagene) Phenotype: Δ(*mcr*A)183, Δ(*mcr*CB-*hsd*SMR-*mrr*)173, *end*A1, *sup*E44, *thi*-1, *gyr*A96, *rel*A1, *lac* [F′, *pro*AB, *lac*^{q}ZΔM15, Tn*10*,(tet^r)] were used to propagate plasmids and select transformants. The pGEM-T (Promega, UK) phagemid vector kit was used to clone PCR products amplified with *Taq* DNA polymerase. This vector is provided pre-digested and with free thymidine 'tails', complementary to the non-template dependent addition of terminal adenosine residues by *Taq*.

Media and growth conditions

M9 minimal salts (l^{-1}; NaH_2PO_4, 6 g, K_2HPO_4, 3 g NH_3Cl, 1 g) supplemented with 10 g l^{-1} glucose was used to propagate bacteria to be used in re-inoculation trials. LB broth (l^{-1}; Tryptone 10 g, Yeast extract 5 g, NaCl 5 g; Miller, 1972), supplemented with 12 g l^{-1} agar (Difco) for solid media, was used to propagate *E. coli* strains.

Mushroom reinoculation trials were conducted at a commercial mushroom production facility utilising the shelf system (Fletcher *et al.,* 1989). Following

inoculation, trial beds were treated in exactly the same way as in normal production with regard to watering, chlorination, harvesting schedule etc.

Pathogenicity trials:

Reinoculation methods: i. Direct injection

An overnight culture (100 µl) of test bacteria was injected, with a 23G hypodermic needle, into the stipe bases of 50 young mushrooms at the 'pin' stage. Care was taken to insert the needle into the centre of the developing stipe and to avoid contamination of the needle with debris from the casing soil. Each injected mushroom pin was marked on the cap with water soluble ink from a marker pen. Control treatments comprised batches of 50 mushrooms injected with M9 + glucose broth and a further 50 marked, uninjected mushrooms. All marked specimens were harvested 7 days later and examined for symptoms and bacterial colonisation as described previously.

Reinoculation methods: ii. Spray inoculation

One litre of an overnight bacterial culture was applied evenly to a 1 m^2 plot of a mushroom bed, at the stage when mycelium could be observed to have freshly colonised the surface of the casing layer. Inoculum was sprinkled onto trial plots with a sterile bottle fitted with a cap drilled with 5x 2.0 mm perforations. 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. This harvesting schedule was repeated for 3 flushes.

Restriction fragment length polymorphism (RFLP) analysis of re-isolates:

Preparation of bacterial genomic DNA

Genomic DNA was prepared from strains by a modification of the method of Pitcher *et al.* (1989). Cells were grown overnight in 5 ml LB broth and recovered

by centrifugation at 10,000 g for 5 min. Cells were resuspended and washed twice in 5 ml cell wash buffer (10 mM Tris-HCl pH 7.5, 10 mM EDTA, 10 mM EGTA, 1 M NaCl). The final pellet was resuspended in 100 µl TE buffer, transferred to a 1.5 ml Eppendorf tube and cells lysed with 0.5 ml GES reagent (5 M Guanidine thiocyanate, 100 mM EDTA, 0.5% [v/v] Sarkosyl); vortexing briefly and incubating at room temperature for 10 min. Lysates were cooled on ice and 0.25 ml cold 7.3 M ammonium acetate added. The tubes were vortexed briefly and incubated on ice for 10 min, after which, 0.5 ml of a 24:1 mixture of chloroform and isoamyl alcohol was added. Tubes were vortexed vigorously and centrifuged at 11,600 g for 10 minutes. The upper phase was transferred to a fresh tube and 0.54 volumes of cold propan-2-ol added. Tubes were inverted several times and the DNA precipitate removed directly by spooling onto a sterile micro-pipette tip. The tip was then carefully agitated in 1 ml 70% (v/v) ethanol and the washed DNA finally dissolved in 100 µl TE. For additional purity, the DNA preparation was subjected to another round of extraction starting at the GES reagent addition stage. Finally, preparations were assessed for purity and yield by spectrophotometry, measuring absorbance at 260 and 280 nm.

Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate, visualise and characterise DNA species. Gels were prepared by adding powdered molecular biology grade agarose (Medium EEO; Boehringer Mannheim) to 1x TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA), at agarose concentrations of between 0.7 and 1.5% (w/v), according to the desired DNA separation range. The mixture was boiled for several minutes in a microwave oven until dissolution was complete. For critical applications, the weight before and after boiling was measured, and the difference due to evaporation made up by addition of distilled water. The molten agarose was

allowed to cool to 55-60˚C and poured into the gel formers of horizontal slab gel apparatus.

Prior to loading and running, DNA samples were mixed with 0.1 volume of loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 40% (w/v) sucrose, 0.1% SDS). Electrophoresis was carried out, usually at room temperature, at up to 100 V until the bromophenol blue marker had migrated to the last 25% of the gel. Following electrophoresis, DNA was visualised by staining with 0.5 mg ml^{-1} ethidium bromide and viewed using a short-wave UV transilluminator (Ultra-Violet Products Inc., USA). Images were digitised and printed using an Image ID system (Appligene Inc.).

Amplification of the 16s ribosomal RNA gene from enteric cluster strain PI98 by the polymerase chain reaction (PCR)

Oligonucleotides corresponding to highly conserved regions of the *E. coli* 16s rRNA gene were synthesised and used as PCR primers according to Lane *et al.* (1985) :

20F (forward primer) : AGTTTGATCCTGGCTCA

519F (forward primer): CAGCAGCCGCGGTAATAC

920R (reverse primer): GCTCAATTCCTTTGAGT

1406R (reverse primer): ACGGGCGGTGTGTGC

(Numbers refer to the corresponding positions of the *E. coli* 16s rRNA gene)

A typical 100 µl PCR reaction comprised 10 µl 10x reaction buffer (Mg^{2+}; Boehringer Mannheim), 5 µl 1%(v/v) Nonidet P-40, 0.5 mM dNTP's, 0.5 µg each primer (a combination of 1 forward and 1 reverse), 10 ng template DNA and 1 U *Taq* DNA polymerase (Boehringer Mannheim). Reactions were overlaid with 50 µl mineral oil and subjected to thermal cycling in a Perkin Elmer 380 thermal cycler, with an initial 4 min at 95˚C, then 30 cycles of 30 s at 95˚C, 30 s at 38˚C

and 80 s at 72˚C. During every cycle, 5 s was added to the elongation step; and the reaction held at 72˚C for 5 min following the final cycle.

Cloning of PCR products

PCR products were subjected to agarose gel electrophoresis, and bands excised and purified using the Geneclean II kit (Bio 101 inc.). The 16s rRNA specific DNA was mixed in a 2:1 molar ratio with pGEM-T vector (Promega) and ligated with T4 DNA ligase, according to manufacturer's instructions.

Transformation of E. coli

Plasmid DNA to be amplified was transformed into competent *E. coli* (strains DH5α or XL1-Blue) based on the method of Sambrook *et al.* (1989). *E. coli* cells were grown overnight at 37˚C in 5 ml LB broth with shaking at 250 rpm. A sample (150 μl) of this culture was transferred to fresh LB broth and incubation continued as before for approximately 2 h until the optical density at 650 nm reached 0.3~0.5. A proportion of this culture (1.5 ml) was transferred to a sterile Eppendorf tube and cells harvested by centrifugation at 11,600 g for 20 s. Cells were resuspended in 500 μl ice cold 50 mM $CaCl_2$ and placed on ice for 10 min, after which, centrifugation was repeated as before. Cells were resuspended in 300 μl cold 50 mM $CaCl_2$ and incubation continued for 20 min.

Competent cells prepared as above were incubated on ice with transforming DNA for 20 min, after which, cells were subjected to a heat shock of 42˚C. Cells were returned to ice for 2 min and 1 ml LB broth at room temperature added. The tubes were then incubated at 37˚C for 1 h, after which cells were concentrated by centrifugation and plated on selective and differential media comprising LB agar supplemented with 50 μg ml^{-1} ampicillin (and an additional 10 μg ml^{-1} tetracycline for *E. coli* XL1-Blue), 20 μg ml^{-1} X-Gal (5-bromo-4-chloro-3-indoyl-β-D-

galactoside) and 0.2 mM IPTG (isopropyl-β-D-thiogalactoside). Plates were then incubated overnight at 37˚C. White colonies containing plasmid DNA with cloned inserts were selected and grown overnight in LB broth supplemented with 50 µg ml^{-1} ampicillin. Plasmid DNA was purified using the Wizard mini-prep kit (Promega) and checked for the presence of cloned inserts by digestion with restriction enzymes *Sac*II and *Pst*I and agarose gel electrophoresis, according to Sambrook *et al*. (1989). The clone, amplified by primers 20F + 920R, was designated pPI20/920 and that by primers 519F + 1406R, as pPI519/1406.

Preparation of biotinylated probe DNA

A sample of pPI20/920 (1.5 µg) containing part of the 16s rRNA gene from strain PI98, was digested with *Sac*II and *Pst*I and insert DNA purified as before. This product was labelled with Biotin-14-dUTP overnight using the components and protocols of the NEBlot Phototope random primer biotin labelling kit (New England Biolabs). Labelled probe was precipitated, washed with 70% (v/v) ethanol and resuspended in 20 µl dH_2O. The yield of probe produced by the above procedure was approximately 1 µg.

Southern Blotting:

Digestion and electrophoresis of bacterial genomic DNA

Genomic DNA from applied and re-isolated bacterial strains (1 µg) was restriction digested to completion overnight. Digests were concentrated and applied to the wells of a 12.5x11 cm slab gel comprising 0.8% (w/v) agarose (Boehringer Mannheim) in 1x TBE buffer. A pre-biotinylated mixture of *Hin*dIII digested lambda DNA and *Hae*III digested ϕX174 DNA (New England Biolabs) was also applied as molecular weight markers. Electrophoresis was carried out at 30 V for 12 h at 4˚C, and the gel stained with ethidium bromide and visualised as above. The choice of restriction enzyme to optimally differentiate between enteric cluster strains was determined empirically.

Transfer and immobilisation of DNA onto Nylon membranes

DNA, following depurination with 0.25 M HCl and denaturation with 0.5 M NaOH, 1.5 M NaCl was transferred to a neutral nylon hybridisation membrane (Hybond N, Amersham) according to Sambrook *et al* (1989), utilising the capillary blotting technique. Transfer buffer was 20X SSC (3 M NaCl, 0.3 M tri-sodium citrate). Subsequently, DNA was crosslinked by baking at 120˚C for 30 min and exposing to UV light for 20 s on a transilluminator (UVP). Filters were stored at -20˚C until required.

Hybridisation with a biotinylated 16s rDNA probe

Filters were placed in a hybridisation bag and prehybridised, fully immersed in a shaking water bath at 68˚C for 1h. Prehybridisation solution comprised 6X SSC, 5X Denhardt's reagent (made from a 50X stock: 1% (w/v) Ficoll 400, 1% (w/v) bovine serum albumin, 1% (w/v) polyvinylpolypyrrolidone), 0.5% (w/v) SDS and 100 µg ml^{-1} denatured, sonicated salmon sperm DNA. The bag was emptied and hybridisation solution added, comprising prehybridisation mix and the denatured biotinylated probe. Hybridisation was carried out overnight at 68˚C.

Stringency washing and detection of DNA hybrids

Following hybridisation, filters were washed with 2X SSC, 0.1% SDS for 5 min at room temperature and then washed twice for 15 min at high stringency (68˚C, 0.1X SSC, 0.1% SDS). The filter was transferred to a fresh bag and hybrids detected with the reagents and according to the protocols supplied with the NEBlot Phototope kit (New England Biolabs). Briefly, the chemiluminescent detection reagent, Lumigen PPD (a phenylphosphate-substituted 1,2-dioxetane) is dephosphorylated by biotinylated alkaline phosphatase, which is attached to the biotin of the nucleic acid hybrids via a streptavidin bridge. This dephosphorylation leads to decomposition of the lumigen with the consequent emission of yellow-green light. Signals were

detected with autoradiography film (Biomax MR, Kodak), with an exposure time of approximately 30 min usually being sufficient.

RESULTS

Following injection with the selected bacteria, all enteric cluster strains produced typical symptoms in the treated mushrooms. Mushrooms injected with *P. fluorescens* produced atypical and mild symptoms, lacking the internal tissue collapse characterised by enteric infections (Table 1). *Ewingella americana* could be isolated in almost pure culture from injected mushrooms and in symptomatic specimens, was present in excess of 10^9 cfu g^{-1}. These strains were phenotypically identical to those applied when examined with API50CHE tests. In the group injected with the *P. fluorescens* strain, bacteriological examination revealed *E. americana* in significant numbers along with the strain applied. The group injected with sterile broth also demonstrated symptoms in 31% of mushrooms, but on examination, was also found to be colonised by *E. americana*.

Table 1. Results of injection trial. 100 µl bacterial culture / broth applied to 50 mushrooms in each treatment.

Strain applied	% symptomatic mushrooms	Symptom severity
PI4	55	+/-
PI74	76	+++
PI98	40	++
PI145	41	+++
PI180	43	+++
Control: M9 + glucose	31	+
Control: uninjected	0	-

Typical disease symptoms were also produced in trial plots sprayed with *E. americana* (Figure 1). As in the injection trial, strain PI74 demonstrated a greater infection frequency than other isolates. The disease also persisted more significantly into the second flush. Mushrooms from the plot treated with the pseudomonad developed atypical symptoms and bacteriological examination also revealed significant colonisation by *E. americana* in these mushrooms along with mixed *P. fluorescens* strains. Bacteria phenotypically identical to the applied pseudomonad were not recovered. The plot treated with broth yielded symptomatic mushrooms, heavily colonised by *E. americana* at 10^7 - 10^9 cfu g^{-1}. Lower counts of *P. fluorescens* were detectable in symptomatic mushrooms treated with broth than in natural infections, but these followed a similar strain distribution to the normal disease pattern.

Table 2. Results of spray reinoculation trial: infection rates following spraying of mushroom beds with 1 l of overnight culture in M9 broth.

Strain applied	% symptomatic mushrooms*		Symptom severity
	1st flush	2nd flush	
PI4	14.8	0.5	+/-
PI74	29.3	3.2	+++
PI98	11.5	0	++
PI145	11.7	0	+++
PI180	11.8	0	+++
Control: M9 + glucose	19.9	0.7	++
Control: Untreated	<1	0	+/-

*Average mushroom yield per m^2 plot was 256 for the first flush and 241 for the second flush. 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).

Figure 1. Mushrooms following spray inoculation trials. Samples were harvested 14 days following inoculation with 1 l of an overnight bacterial culture in M9 broth. During this time, plots were treated to normal watering and chlorination regimen.

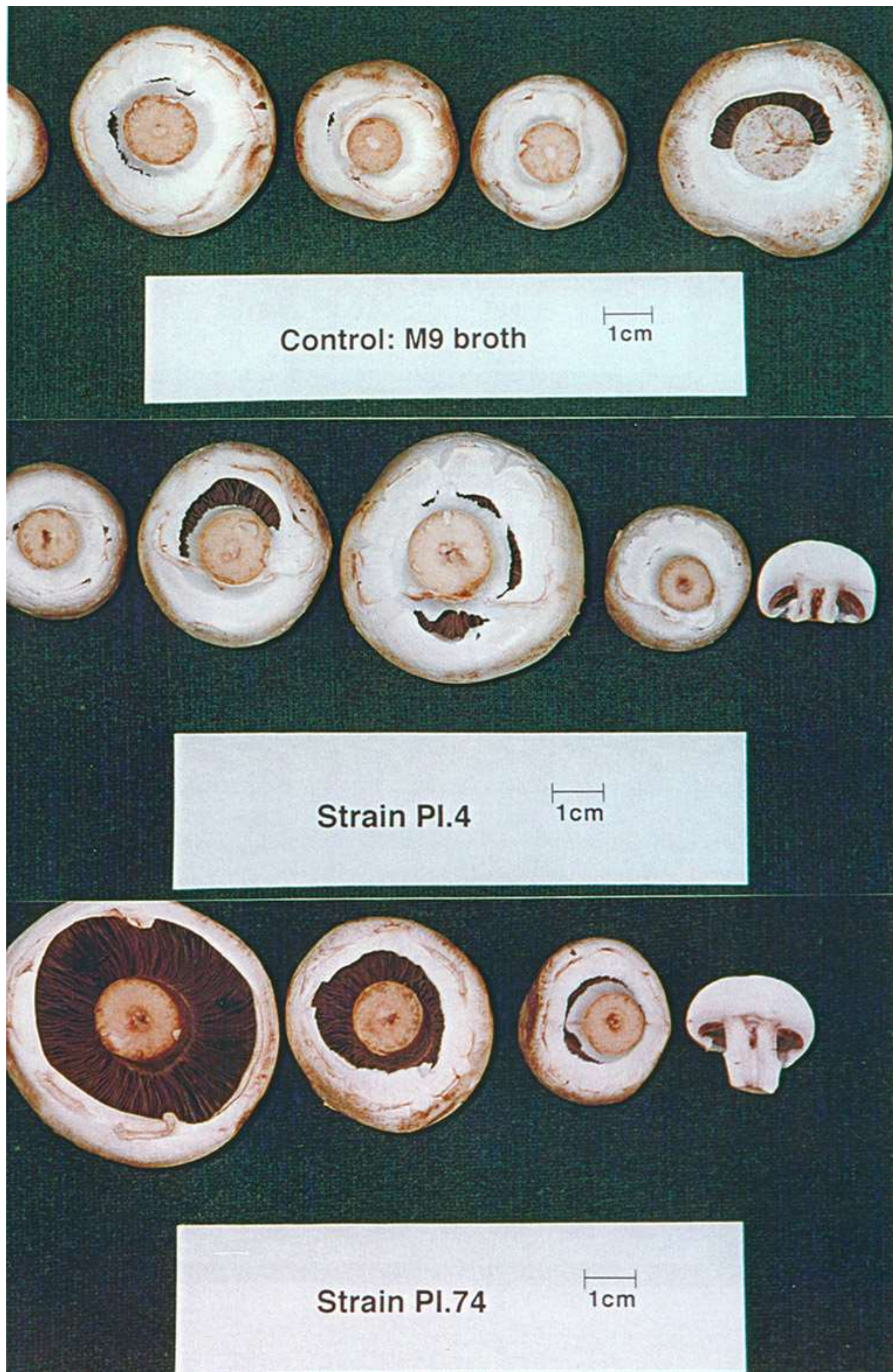
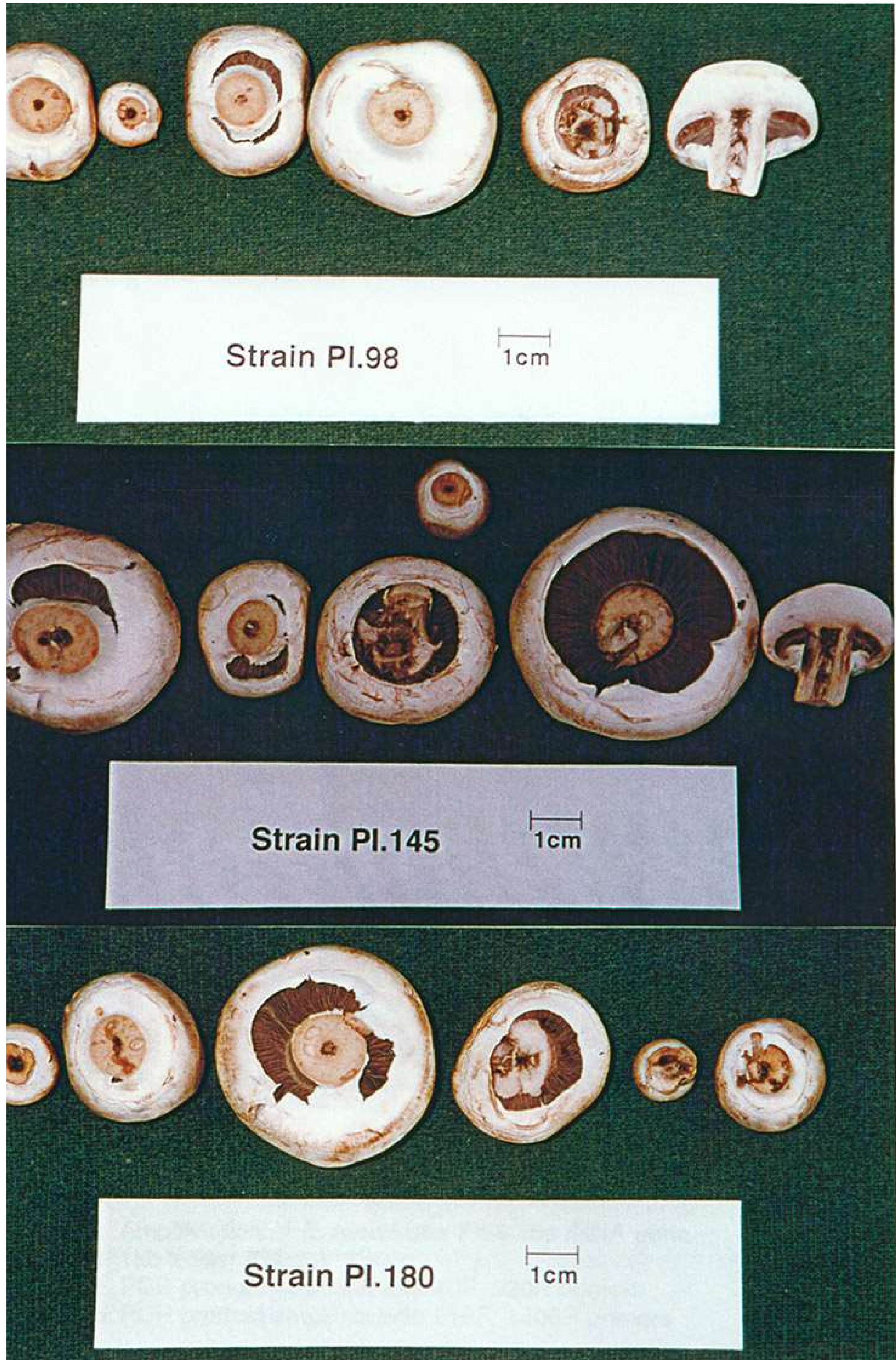


Figure 1 cont.



Molecular fingerprinting and confirmation of Koch's postulates:

The 16s ribosomal RNA gene from stipe necrosis strain PI98 (*E. americana*) was successfully amplified using the two overlapping sets of primers (Figure 2). Both sets of primers yielded PCR products of approximately 900 bp, which was expected from their conserved positions relative to the corresponding *E. coli* gene. Final confirmation that this was 16s rDNA was obtained by DNA sequencing (see Chapter 4).

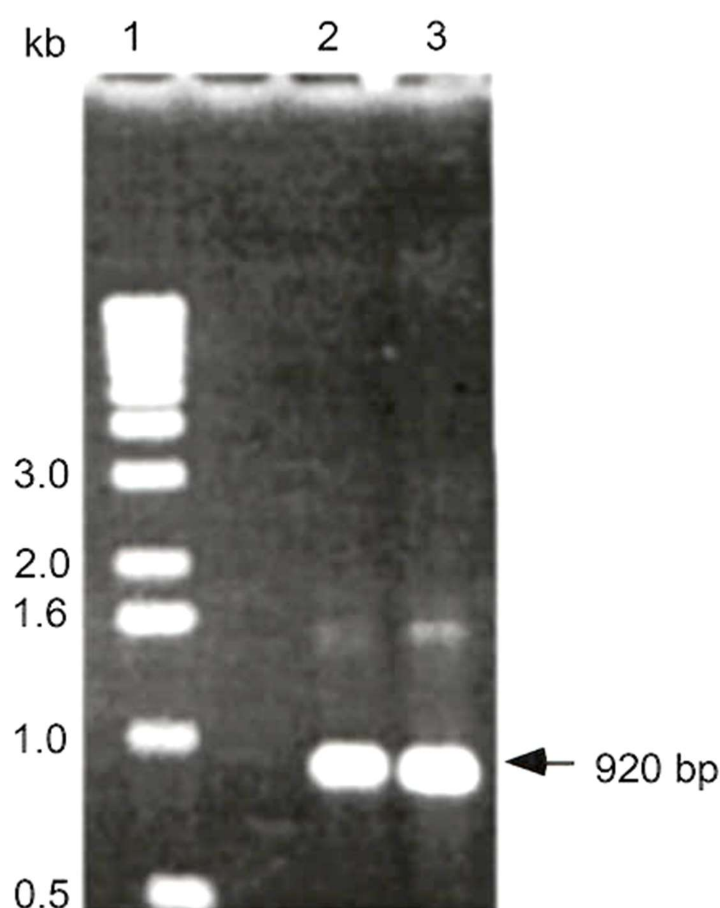


Figure 2. Amplification of *E. americana* PI98 16s rRNA gene.
Lane: 1. 1kb ladder (Gibco)
2. PCR product amplified with 20F, 920R primers
3. PCR product amplified with 519F, 1406R primers

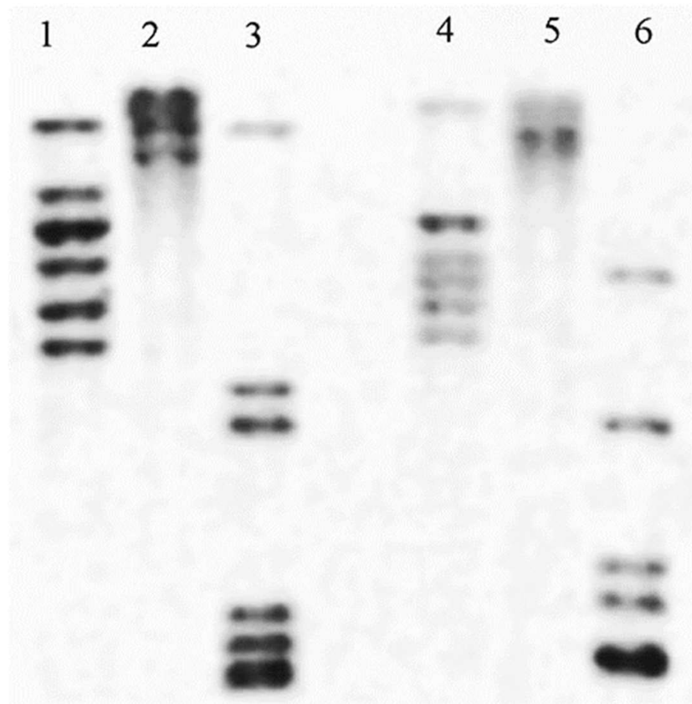


Figure 3. RFLP optimisation; Southern blot probed with *E. americana* 16s rRNA gene. Lane 1: PI74 genomic DNA restricted with *Hind*III + *Eco*R1, Lane 2: *Pst*I, Lane 3: *Pvu*II, Lane 4: PI145 restricted with HindIII + *Eco*R1, Lane 5: *Pst*I, Lane 6: *Pvu*II. Molecular weights not shown.

Strains PI74 and PI145 were compared in an optimisation procedure to find a restriction enzyme that produced easily distinguishable patterns in RFLP blots. *Puv*II was found to produce a wide spread of bands in the trial RFLPs that readily differentiated the two strains by simple visual examination (Figure 3). This enzyme was used in subsequent RFLPs to monitor applied and reisolated strains in the Koch's postulate trials.

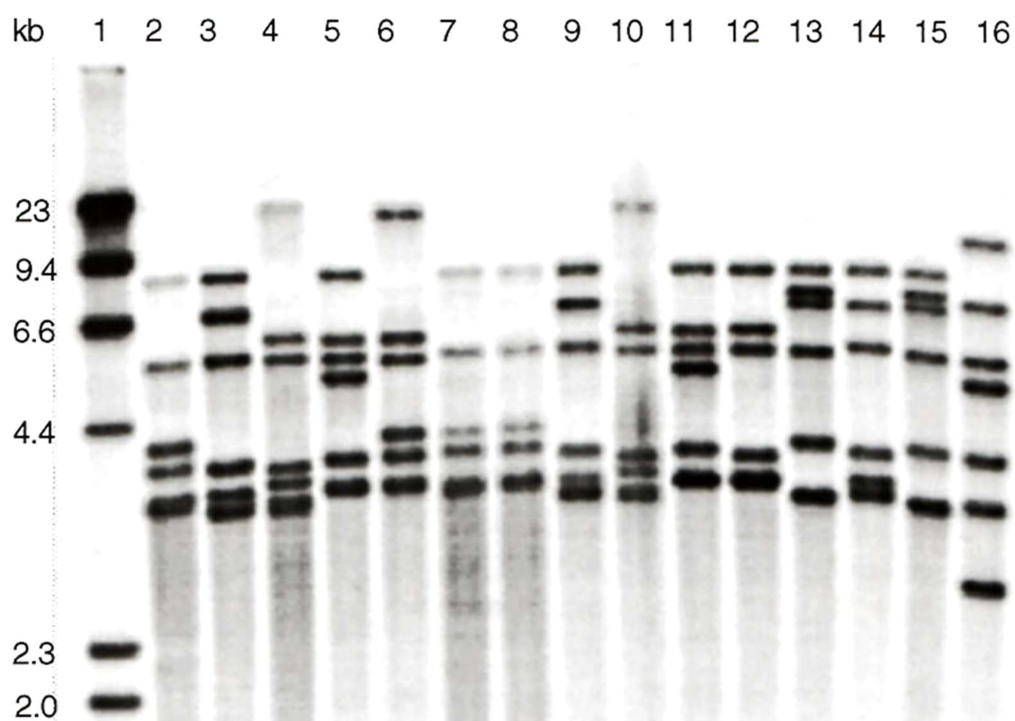


Figure 4. RFLP: Genomic DNA digested with *Pvu*II, probed with the *E. americana* 16s rRNA gene. For each treatment, 3 mushrooms were homogenised and plated on differential medium as described previously. A single chitinolytic colony was randomly selected from each re-isolation plate for RFLP analysis. Depicted profile contains a representative selection of recovered strains.

Lane:
1: Pre-biotinylated mixture of *Hin*dIII digested lambda DNA and *Hae*III digested ϕX174 DNA.
2: PI74
3: PI98
4: PI145
5: PI180
6: *E. americana* NCTC 12157
7: Reisolate from 1st. flush treated with PI74
8: Reisolate from 2nd flush treated with PI74
9: Plot treated with PI98
10: Plot treated with PI145
11: Plot treated with PI180
12: Plot treated with M9 broth + glucose
13: Plot treated with M9 broth + glucose
14: Plot treated with M9 broth + glucose
15: Plot treated with M9 broth + glucose
16: Chitinolytic enteric bacterium isolated from peat sample

RFLP studies readily differentiated the strains applied in the Koch's postulate trials. The technique also confirmed a close relationship between the strains, since they shared several common bands, although only one band, of approximately 5 kb was common to all strains. The RFLP profile of the type strain of *E. americana* (ATCC 12157) displayed several bands in common with stipe necrosis strains, indicating a close taxonomic affiliation. The technique demonstrated that recovered strains following deliberate infection were identical to those applied. Symptomatic mushrooms examined from the plot treated with M9 broth yielded *E. americana* strains demonstrating a wide range of RFLP profiles. One isolate (lane 14) had a profile identical to strain PI94, but other isolates were clearly differentiated from the applied strains. A strain isolated from casing material (RFLP lane 16) displaying a fermentative and chitinolytic phenotype, was shown to have a highly dissimilar RFLP to *E. americana* strains, and in API tests was found not to be *E. americana*.

DISCUSSION

The results presented provide strong evidence for the involvement of the enteric-cluster isolates in mushroom internal stipe necrosis. The fact that these isolates could not be differentiated by biochemical tests and displayed common bands in RFLP studies probably indicates that they are probably all strains belonging to a single species, which is most likely to be related to, or be synonymous with, *Ewingella americana*. It can be demonstrated that following deliberate infection of mushrooms with *E. americana*, symptoms identical to natural internal stipe necrosis can be reproduced. Mushrooms injected with, and plots sprayed with, a stipe necrosis mushroom-derived *P. fluorescens* isolate failed to produce typical symptoms. These results conflict with the conclusions of Richardson (1993), who claimed that

mushroom stipe necrosis was caused by a fluorescent pseudomonad (full phenotypic and taxonomic descriptions were not given). The experimental procedures, however, were severely limited, in that a single injection trial with a single isolate was conducted on an extremely small mushroom sample (12). In addition, the author claimed to have injected the mushroom bases with 0.5ml of bacterial suspension. This injected volume was found in this study, to invariably result in severe mechanical damage, thereby increasing the likelihood of post injection trauma and secondary infection. In addition, only King's B medium was used for primary bacterial isolation, which in this study was found to be inadequate to differentiate the range of *P. fluorescens* colonial types and other bacteria present in stipe necrosis affected mushrooms (see Chapter 2).

The injection trial carried out in this study resulted in between 40 and 76% of harvested mushrooms showing typical symptoms. Strain PI74 appeared to be more pathogenic than the other *E. americana* strains tested and this phenomenon was repeated in the spray inoculation trial. The reasons for this difference are currently uncertain, but warrant further investigation. This differential virulence may provide the means to identify important properties of the bacteria that result in pathogenesis. Very few stipe necrosis affected mushrooms were harvested at the second flush during the spray infection trial. This was probably indicative of the effectiveness of normal chlorination regimen in suppressing the disease-causing bacteria in colonised casing. This phenomenon could also indicate that thorough chlorination of colonised casing at the time of primordium formation could be important in preventing disease development. Indeed, outbreaks could be caused by faulty chlorination practices, since the data from broth application experiments strongly suggests that *E. americana* is probably endemic.

A relatively high inoculum was used in these experiments, although these were lower than that used by Richardson (1993). In the case of the injection method,

useful data would be provided by conducting a dose-response trial in order to determine the minimal bacterial number necessary to cause symptom development. This trial should also be conducted using suspensions of bacteria in non-nutritive buffer in order to eliminate the broth stimulation effect encountered here. In the case of spray trials, however, the inoculum size utilised here was typical of studies in mushroom diseases. In the case of *P. tolaasii*, large inocula are necessary in artificial inoculation experiments, where the survival rates of the pathogen in mushroom casing is low (Wong & Preece, 1982). Such survival data would be invaluable for ISN strains, in particular, in situations of normal mushroom cultivation; especially with regard to prophylactic chlorination and the composition of the normal microbial flora of the casing.

The development of symptomatic mushrooms in trials using sterile broth could present complications in the interpretation of Koch's postulates. However, bacteria recovered from these mushrooms were shown, in phenotypic tests, to be *E. americana* and were clearly differentiated from strains applied in deliberate infections by RFLP analysis. The range of strains discovered to be naturally present in the trial plots additionally points to the endemic nature of *E. americana* in mushroom culture. The long-term stability of RFLP profiles, along with other molecular strategies for strain typing, should be critically examined in the light of the findings of Hall (1994) and others. It is probably impossible to apply a "gold standard" for bacterial strain tracking, due to the inherently mutable nature of the bacterial genome. However, the use of sufficiently large sample sizes and effective controls in experiments means that we can probably disregard variation produced by infrequent and random genome rearrangements in trial strains. In this study, the vast majority of RFLP-types recovered from plots were found to be identical to the applied strain, indicating a low frequency of mutation. Variants are most likely to

be due to infection by endemic strains stimulated by residual nutrients in the broth-based inoculum.

RFLP analysis has confirmed that the strains applied in these trials are identical to the majority of those recovered in mushrooms displaying the typical symptoms of internal stipe necrosis. Taking into account the prevalence data presented in Chapter 2, it can be concluded that *Ewingella americana* satisfies the requirements of Koch's postulates as the causative agent of mushroom internal stipe necrosis.

CHAPTER 4
Taxonomy of the stipe necrosis causative agent

INTRODUCTION

The enteric group of isolates identified as significant in Chapter 2 and proven to be the cause of ISN as described in Chapter 3, were presumptively classified as probable *Ewingella americana* Grimont. This identification was based on conventional biochemical profiling and a commercial bacterial identification kit (API20E - BioMérieux). The commercial biochemical profile system is inherently probabalistic (Anon, 1989) and, as discussed previously several significant properties of ISN strains were different from the published descriptions of *E. americana* (Grimont *et al.,* 1983; Farmer III *et al.*, 1985; Ewing, 1986); notably, production of chitinase and Tween 80 esterase. Additionally, this species was formally known only from clinical specimens (Gross & Holmes, 1990). It was necessary, therefore, to fully examine the taxonomy of ISN strains, either to confirm identity as *E. americana,* or possibly to propose the ISN strains as a new species of *Enterobacteriaceae.* The latter idea was supported by the fact that these strains showed phenotypic similarity to certain plant pathogenic *Erwinia* sp. and *Enterobacter agglomerans* / *Pantoea* sp., but were not synonymous with any previously described mushroom or plant associated species when compared using conventional tests (Holt *et al.,* 1994).

To confirm the taxonomy of ISN strains, tests to supplement the conventional biochemical profiles were necessary. Several automated systems now exist to analyse various chemical components of bacteria, usually by means of chromatography of certain cellular components. Pyrolysis mass spectrometry has

proved to be a rapid, sensitive and reproducible method for strain typing and identification (Freeman *et al*., 1991). The method is based on the principle of oxidising whole bacterial cells and analysing the products of combustion by mass spectrometry. Analysis of cellular fatty acid methyl esters (FAMEs) is a similar approach (Mukwaya & Welch, 1989; Stead, 1992; Stead *et al.,* 1992), where fatty acids are extracted from bacterial cells with solvent, and the products analysed by gas chromatography. Both methods are reliant on standardisation of bacterial cultural conditions and computerisation in the interpretation of the profiles obtained. For identification purposes, these methods are also dependant on the level of completeness of the libraries with which the software compares the experimental data.

Molecular techniques applied to bacterial taxonomy

Besides providing powerful tools for epidemiological studies, the techniques of molecular biology have been applied successfully to higher level taxonomy; that is, the naming and definition of bacterial systematic groupings. Such methods have assisted in elucidating the structure underlying the interrelationships of species within genera as, for example, in *Pseudomonas* (Palleroni *et al.,* 1973; Champion *et al.,* 1980; Palleroni, 1984; Palleroni, 1993) and *Serratia* (Dauga *et al.,* 1990). Attempts have been made to use certain techniques and markers to work out the phylogeny of all bacteria. This is based on the theory that certain ubiquitous, conserved genes might serve as evolutionary indicators, when examined at the sequence level. Several of these "molecular clocks" have been examined, for example, elongation factor EF-2 / EF-G (Cammarano *et al*., 1992) and glutamine synthetase (Pesole, *et al*., 1991). By far the most widely accepted markers, however, are the ribosomal RNA genes (rDNA) (Woese, 1994; Fox & Stackbrandt, 1987); and there exists today a large database of available rDNA sequences, in particular,

small sub-unit or 16S rDNA (Gutell, 1993). Ribosomal RNA data have been used to determine close and distant relationships and have even been applied to the naming and classification of uncultured microorganisms (Ward *et al*., 1990). Members of a distinct species are normally expected to display a level of 16S rDNA sequence identity approaching 100%, with allowances being made for possible sequencing errors. Sequence identity of rDNA genes, however, may not necessarily guarantee that a strain under examination may be assigned to a certain species (Fox *et al*., 1992). This is especially true of recently diverged species.

Measurement of DNA hybridisation between strains is the single most definitive tool for delineating a species (Kenneth, 1995). Such data has also been a central factor in rationalising current, or elevating new genera. An example of this is the renaming of the formally heterogeneous species *Enterobacter agglomerans* as *Pantoea agglomerans* and recognition of *Pantoea dispersa* as a discrete species within this former assemblage (Gavini *et al*., 1989). The level of genomic homology required to define a species boundary has been accepted as being 70% (Wayne *et al*., 1987). This figure was arrived upon following exhaustive surveys of existing, well-defined species, and appears to correlate well with conventional methods of species delineation.

Numerous approaches to the estimation of DNA hybridisation values have been applied (reviewed by Kurtzman, 1993). The reproducibility and correlation of certain procedures was critically examined by Grimont *et al*. (1980), who found significant differences in absolute values for relative binding ratios (RBR) among two variants of the S1 nuclease method (Grimont *et al*., 1979) and the hydroxyapatite method (Brenner, 1978). These differences, however, were dependant on hybridisation stringency and could be normalised by application of a small correction. The hydroxyapatite method has been proposed as the yardstick with which other approaches are correlated; probably for historical reasons.

However, the method carries with it the disadvantages that considerable quantities of radioactive material are utilised, with the concomitant problems of waste disposal and safety and that each sample must be measured separately in a scintillation counter. Filter hybridisation methods have the advantage that multiple target DNA's may be immobilised on a single membrane and require less labelled tracer DNA. The procedure adopted in this study is a non-radioactive, chemiluminescent version of the filter hybridisation technique, which is a development of the colorimetric method of Jahnke (1994).

MATERIALS AND METHODS

Physiological profile of the stipe necrosis agent.

In addition to screening tests adopted in Chapter 2, selected strains resembling *E. americana* were analysed using API 20E and API 50 CHE (BioMérieux) panels according to manufacturer's instructions. Panels were incubated at 28˚C for the recommended time, and the resulting profiles identified using the API analytical profile index (Anon, 1989) and computer identification service. The type strain of *E. americana NCTC* 12157 (= ATCC 33852) was obtained from the National Collection of Type Cultures (Colindale, UK) and used as reference.

Analysis of cellular fatty acid content of ISN strain PI98

Gas chromatography of cellular FAMEs from strain PI98 was carried out by Dr. D.E. Stead of the Central Science Laboratory; National Collection of Plant Pathogenic Bacteria (Harpenden, UK). Profiles were compared with those from all strains held at the above culture collection (NCPPB library, Rev. 3.0) and a commercial profile library (TSBA, TSBA+ libraries; Microbial ID Inc., Newark, D.E., USA)

Antibiotic sensitivity testing

Disc diffusion sensitivity tests were performed on Isosensitest agar (Oxoid) plates according to Phillips (1991), using *Staphylococcus aureus* (Oxford strain) in Stokes control method. Antibiotic multi-disks were purchased from Mast Diagnostics UK Ltd and applied to plates seeded with bacteria to produce a dense but non-confluent growth. Plates were then incubated overnight at 28˚C.

Electron microscopy of stipe necrosis strain PI98 (Negative stain).

An overnight culture of bacteria (100 µl) was added to 10 ml fresh LB broth and incubation continued with shaking for approximately 2 h. During this time, 25 µl samples were taken at 0.5 h intervals and observed by light microscopy until most of the bacterial population appeared actively motile. Cells were then harvested by centrifugation for 5 min at 6000 g and gently resuspended in 1 ml 0.5 M sucrose, 0.15 M Tris-HCl pH 8.0. A freshly prepared solution of 10mg ml^{-1} hen egg white lysozyme (100 µl) and 100 µl 0.1 M EDTA pH 7.0 was added. The cells were then incubated on ice for 10 min, after which, 5 µl of treated cells were gently pipetted onto a formvar coated copper electron microscope grid. The grids were left to settle for 1 min and then washed with 100 µl dH_2O. Excess liquid was blotted with Whatman 3MM paper and 1 drop of 2% (w/v) phosphotungstic acid added. Grids were washed 30 s later, blotted twice as before and left to air-dry. Specimens were then observed with a JEOL 100B transmission electron microscope.

Sequencing of the 16S ribosomal RNA gene from stipe necrosis strain PI98.

The 16S rRNA gene of strain PI98, cloned into the pGEM-T vector was produced as described in Chapter 3. The gene was also excised from the T-vector and inserted in the opposite orientation relative to the vector *lac*Z promoter in the phagemid vector pBluescript II SK+ (Stratagene). Subclones were produced so that the complete sequence of this gene from positions 20 - 1406 (relative to the corresponding positions of the *E. coli* 16S rRNA gene) could be determined for both DNA strands.

Preparation of Single stranded DNA for sequencing.

Five ml of 2 x LB broth in a cotton wool capped boiling tube was inoculated with 100 µl of an overnight culture of *E. coli* strain XL1-Blue MRF$^-$ harbouring phagemid DNA, this was incubated for 2 h at 37˚C with shaking at 300 rpm. Helper phage VCSM13 (Stratagene, UK) was added at an approximate multiplicity of infection of 10 (approximately 25 µl) and incubation continued for 2 h, after which, kanamycin was added at 70 µg ml^{-1} and incubation continued overnight. Cells were then removed by centrifugation for 10 min at 17,000 g. The supernatant was placed on ice and phage particles precipitated by the addition of 1 ml cold phage precipitation buffer (30% (w/v) polyethylene glycol (PEG 8000), 3 M NaCl). After 30 min, the phage particles were collected by centrifugation at 10,000 g for 15 min and the supernatant discarded. Tubes were centrifuged again and the last traces of supernatant aspirated. The phage pellet was then resuspended in 0.7 ml phage lysis and binding buffer, supplied with the QIAprep Spin M13 kit (Quiagen UK) and the mixture passed twice through the kit binding column. Further purification and elution of DNA was according to the protocol provided by the manufacturer. DNA was finally checked for purity and yield by agarose gel electrophoresis.

DNA sequencing.

Single stranded DNA from suitable subclones was subjected to dideoxynucleotide, chain termination sequencing (Sanger *et al*., 1977) using the Sequenase version 2.0 DNA sequencing kit (Amersham, UK), according to manufacturer's recommendations, using the universal M13 forward sequencing primer and [α-^{35}S]dATP as radioactive label. Sequencing gels were prepared using Sequagel (Flowgen, UK) reagents and glycerol tolerant electrophoresis buffer (20x stock, l^{-1}: Tris base 216 g, Taurine 72 g, EDTA 4 g); gels were run at 55 W constant power in a Flowgen VM4133 vertical gel apparatus. Following autoradiography, gels were read at least twice in order to minimise reading and typographical errors.

Sequence information was assembled using the Genejocky DNA sequence analysis computer package and sequence homology with other bacteria assessed by conducting searches of the EMBL nucleotide sequence data library using the FASTA (Pearson & Lipman, 1988) similarity search and alignment program.

Determination of DNA hybridisation values.

DNA hybridisation data was gathered by measuring relative binding of stipe necrosis strain PI98 (NCPPB 3905) genomic DNA against DNAs prepared from a selection of reference type strains obtained from the National Collection of Plant Pathogenic Bacteria (NCPPB), the National Collection of Type Cultures (NCTC) and the National Collection of Industrial and Marine Bacteria (NCIMB).

Preparation of a genomic probe.

Genomic DNA (1.5 µg) from stipe necrosis strain PI98 was diluted, boiled and labelled with Biotin-14-dATP overnight according to manufacturer's instructions. The estimated final yield of probe DNA following this procedure was 1.0 µg.

Immobilisation of test DNA's.

Test DNA (1 µg) was diluted with alkali transfer buffer (1.5 M NaCl, 0.25 M NaOH) to a final volume of 200 µl. Samples were then boiled for 5 min and snap chilled on

ice. Samples were applied by vacuum to Hybond N (Amersham, UK), a non-charged nylon hybridisation membrane, prewetted with alkali transfer buffer. This was accomplished using a 96 well microfiltration 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. Following filtration, each well was washed with 500 µl 6x SSC. The apparatus was then carefully disassembled and the membrane washed in 6x SSC. The membrane was then baked for 30 min at 120˚C and crosslinked for 20 s using a shortwave UV transilluminator (UVP inc., USA).

Hybridisation and quantitation.

Hybridisation was carried out as described previously with 2 x 15 min washes at high stringency (68˚C, 0.1 x SSC, 0.1% SDS). Hybrids were then detected using the Phototope detection kit (New England Biolabs). Chemiluminescent hybridisation signals were quantified using a Bio-Rad phosphor imaging system fitted with a gel cassette designed for chemiluminescent detection. This equipment is claimed to have a 10-fold increase in bandwidth as compared to X-ray film, which is likely to eliminate saturation problems encountered when attempts are made to quantify autoradiograph images. Processed data from the Bio-Rad imager was adjusted for background using data from the blank wells and relative hybridisation assessed by comparing data from test wells to that of fully homologous wells containing DNA from strain PI98.

RESULTS

FAME profile of ISN strain PI98

(Table 1) ISN strain PI98 did not give a positive, or significant match with any strain held in the libraries screened. Closest matches were with other enteric bacteria; *Morganella morganii*, *Xenorhabdus luminescens* and *Yersinia pseudotuberculosis*. *Ewingella americana* was not represented in the versions of the libraries tested and so this possibility could not be excluded.

Table 1. Fatty acid composition profile of strain PI98.

ECL*	Fatty acid	% total
12.000	12:0	3.52
13.001	13:0	0.34
13.959	Unknown 13.961	0.70
13.999	14:0	2.41
14.503	Unknown 14.503	0.95
15.000	15:0	2.05
15.490	? 14:0 3OH/16:1 ISO I	8.80
15.818	16:1 w7c	1.99
16.000	16:0	34.45
16.891	17:0 CYCLO	28.49
16.999	17:0	1.55
17.822	? 18:1 w7c/w9t/w12t	4.01
17.997	18:0	0.81
18.901	10:0 CYCLO w8c	7.33

*ECL = fatty acid equivalent chain length; % total = relative amount of that acid estimated by integration of the individual chromatogram peak area. Most probable fatty acid identity is given in cases indicated by ?. This situation arises where 2 or more acids are known to co-elute. (Analysis kindly provided by J.E. Sellwood & D.A. Stead; National collection of Plant Pathogenic Bacteria).

Phenotypic characterisation of ISN strains

The API 20E panel identified ISN strains as *Ewingella americana* (code number 1205101) with 99.1% confidence (Manufacturers statistic; Anon 1989). The API50CHE panel (Table 2) again identified strains as *E. americana* with 99.8% confidence. Variation between strains using these tests was not observed up to 48 h incubation time, after which, some tests, such as 2 ceto-gluconate did demonstrate some variation. Other tests such as gluconate and L-fucose utilisation gave weak/intermediate responses at 72 h incubation time. The reliability of these individual tests, therefore, might be questioned. The type strain of *E. americana* (NCTC 12157) also gave an identical profile to mushroom-derived strains in API50CHE tests. This strain was also found to be positive for chitinase, when examined using LB agar supplemented with RBV stained CM-chitin, producing similar clearing zone sizes to ISN strains (Data not shown).

Variation was observed among ISN strains with regard to sensitivity to antibiotics. 100% of strains were sensitive to ampicillin, ticarcillin, tetracycline, nalidixic acid and gentamicin. Resistance to sulphonamide was observed in 90% of strains, to trimethoprim in 74% of strains, to co-trimoxazole in 16% of strains and to nitrofurantoin in 11% of strains.

Electron microscopy of ISN strain PI98

(Figure 1) The negatively stained bacteria demonstrated peritrichous flagellation, typical of the *Enterobacteriaceae,* with flagella number varying between 2 and 7. Some specimens also demonstrated possible fimbriae-like cellular projections, although these may represent preparation artifacts (Figure 1, b).

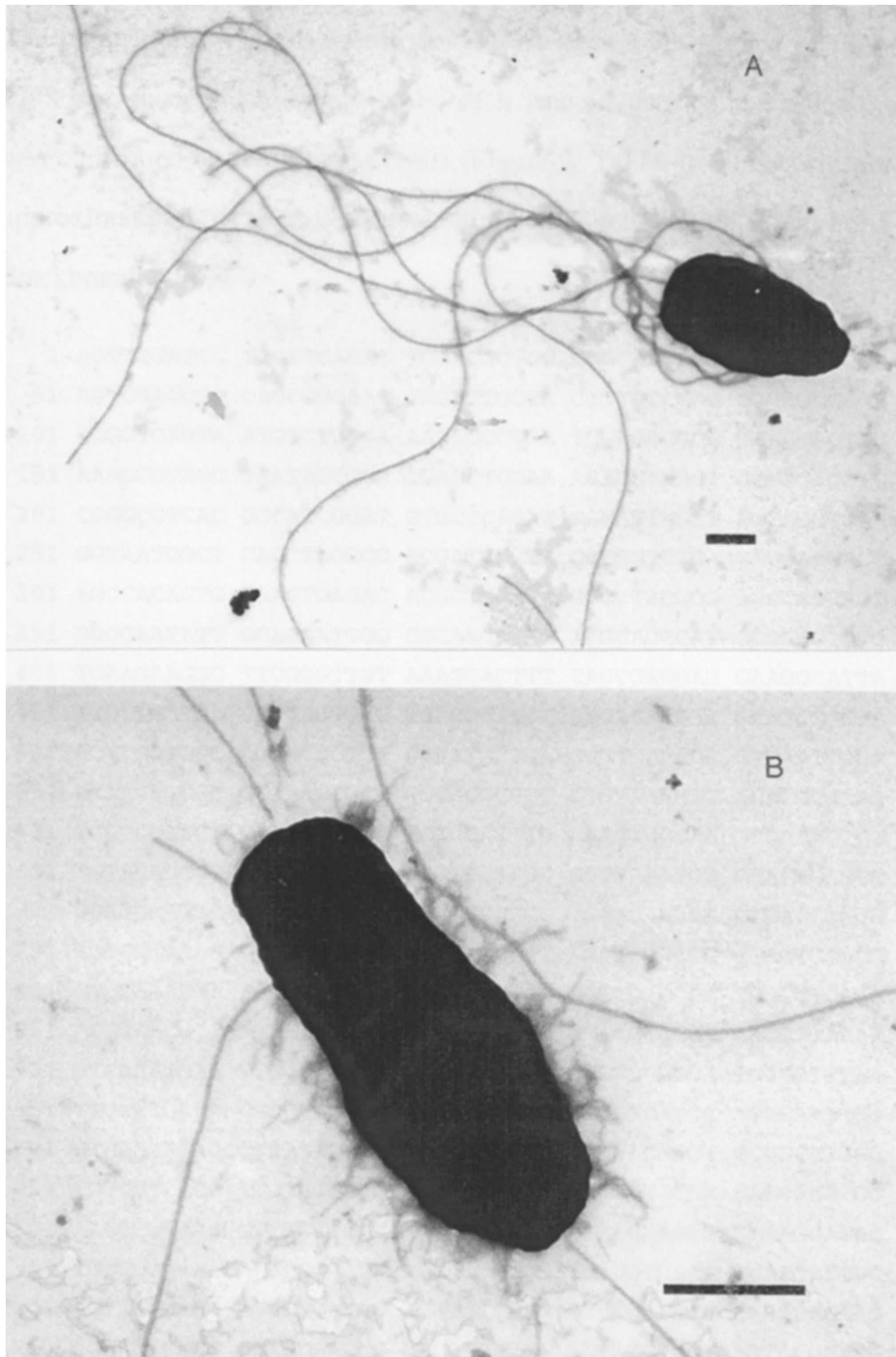
API 50CHE	24 h			48 h			72 h		
Test	1	2	3	1	2	3	1	2	3
Control	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	+	+	+	+	+	+
Erythritol	-	-	-	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	-	+	+	+
L-Arabinose	-	-	-	-	-	-	+	+	+
Ribose	+	+	+	+	+	+	+	+	+
D-Xylose	-	-	-	-	-	-	+	+	+
L-Xylose	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-
β Methyl-xyloside	-	-	-	-	-	-	-	-	-
Galactose	+	+	+	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+	+	+
L-Sorbose	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-
Mannitol	+	+	+	+	+	+	+	+	+
Sorbitol	-	-	-	-	-	-	+	+	+
α Methyl-D-mannoside	-	-	-	-	-	-	-	-	-
α Methyl-D-glucoside	-	-	-	-	-	-	-	-	-
N acetyl glucosamine	+	+	+	+	+	+	+	+	+
Amygdalin	-	-	-	-	-	-	-	-	-
Arbutin	+	+	+	+	+	+	+	+	+
Esculin	+	+	+	+	+	+	+	+	+
Salicin	+	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+
Melibiose	-	-	-	-	-	-	-	-	-
Saccharose	-	-	-	-	-	-	-	-	-
Trehalose	+	+	+	+	+	+	+	+	+
Inulin	-	-	-	-	-	-	-	-	-
Melezitose	-	-	-	-	-	-	-	-	-
D-Raffinose	-	-	-	-	-	-	-	-	-
Amidon	-	-	-	-	-	-	-	-	-
Glycogen	-	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-	-
β Gentiobiose	+	+	+	+	+	+	+	+	+
D-Turanose	-	-	-	-	-	-	-	-	-
D-Lyxose	-	-	-	-	-	-	+	+	+
D-Tagatose	-	-	-	-	-	-	-	-	-
D-Fucose	-	-	-	-	-	-	+	+	+
L-Fucose	-	-	-	-	-	-	+	+	±
D-Arabitol	+	+	+	+	+	+	+	+	+
L-Arabitol	-	-	-	-	-	-	-	-	-
Gluconate	-	-	-	±	-	-	±	±	±
2 ceto-gluconate	±	±	-	+	+	+	+	+	+
5 ceto-gluconate	+	+	+	+	+	+	+	+	+

Table 2.
Results of API50CHE tests on strains:

1. ISN strain PI98
2. ISN strain PI180
3. *E. americana* NCTC 12157

Panels were incubated at 28˚C and examined at 24h, 48h and 72h

+ = positive test (acid produced)
- = negative test (no acid)
± = intermediate (weak) response



Bar = 1μm

Figure 1. Transmission electron micrographs of negatively stained log phase *E. americana* PI98 cells.

16S rRNA gene sequencing

The major portion (1398 bp) of the 16S ribosomal RNA gene of ISN strain PI98 was successfully amplified by PCR (see Chapter 3) and cloned, which was confirmed by sequence analysis (Figure 2; Table 4). This corresponds to approximately 90% of the equivalent E. *coli* gene length (1550 bp; Fox & Stackbrandt, 1987).

```
   1 AGTTTGATCC TGGCTCAGAT TGAACGCTGG CGGCAGGCCT AACACATGCA
  51 AGTCGAGCGG CAGCGGGAAG TAGCTTGCTA CTTTGCCGGC GAGCGGCGGA
 101 CGGGTGAGTA ATGTCTGGGA AACTGCCTGA TGGAGGGGGA TAACTACTGG
 151 AAACGGTAGC TAATACCGCA TGACCTCGAA AGAGCAAAGT GGGGGACCTT
 201 CGGGCCTCAC GCCATCGGAT GTGCCCAGAT GGGATTAGCT AGTAGGTGAA
 251 GGTAATGGCT CACCTAGGCG ACGATCCCTA GCTGGTCTGA GAGGATGACC
 301 AGCCACACTG GAACTGAGAC ACGGTCCAGA CTCCTACGGG AGGCAGCAGT
 351 GGGGAATATT GCACAATGGG CGCAAGCCTG ATGCAGCCAT GCCGCGTGTG
 401 TGAAGAAGGC TTCGGGTTGT AAAGCACTTT CAGCGAGGAG GAAGGCATTA
 451 AGGTTAATAA CCTTAGTGAT TGACGTTACT CGCAGAAGAA GCACCGGCTA
 501 ACTCCGTGCC AGCAGCCGCG GTAATACGGA GGGTGCAAGC GTTAATCGGA
 551 ATTACTGGGC GTAAAGCGCA CGCAGGCGGT TTGTTAAGTC AGATGTGAAA
 601 TCCCCGAGCT TAACTTGGGA ACTGCATTTG AAACTGGCAA GCTAGAGTCT
 651 TGTAGAGGGG GGTAGAATTC CAGGTGTAGC GGTGAAATGG CTAGAGATCT
 701 GGAGGAATAC CGGTGGCGAA GGCGGCCCCC TGGACAAAGA CTGACGCTCA
 751 GGTGCGAAAG CGTGGGGAGC AAACAGGATT AGATACCCTG GTAGTCCACG
 801 CTGTAAACGA TGTCGATTTG GAGGTTGTGG GCTTGACCCG TGGCTTCCGG
 851 ACGTAACGCG TTAAATCGAC CGCCTGGGGA GTACGGCCGC AAGGTTAAAA
 901 CTCAAATGAA TTGACGGGGG CCCGCACAAG CGGTGGAGCA TGTGGTTTAA
 951 TTCGATGCAA CGCGAAGAAC CTTACCTACT CTTGACATCC AGAGAATTCG
1001 CTAGAGATAG CTTAGTGCCT TCGGGAACTC TGAGACAGGT GCTGCATGGC
1051 TGTCGTCAGC TCGTGTTGTG AAATGTTGGG TTAAGTCCCG CAACGAGCGC
1101 AACCCTTATC CTTTGTTGCC AGCACGTCAT GGTGGGAACT CAAAGGAGAC
1151 TGCCGGTGAT AAACCGGAGG AAGGTGGGGA TGACGTCAAG TCATCATGGC
1201 CCTTACGAGT AGGGCTACAC ACGTGCTACA ATGGCATATA CAAAGAGAAG
1251 CGAACTCGCG AGAGCAAGCG GACCTCATAA AGTATGTCGT AGTCCGGATT
1301 GGAGTCTGCA ACTCGACTCC ATGAAGTCGG AATCGCTAGT AATCGTAGAT
1351 GAGAATGCTA CGGTGAATAC GTTCCCGGGC CTTGCACACA CCGCCCGT
```

Figure 2 Nucleotide sequence of the amplified 16S rRNA gene of *Ewingella americana* strain PI98 (NCPPB 3905). Deposited in Genbank under accession number U29438 and in the EMBL nucleotide sequence database as X88848.

Table 4. List of most homologous bacterial species to presumptive *E. americana* strain PI98, based on 16S rRNA data (1398bp). Rankings were generated by the FASTA program using the EMBL prokaryotic sequence databank and have been sorted by the optimised alignment score. The species giving the best match to ISN strain PI98 was *Rahnella aquatilis,* which was found to display 97.1% sequence identity.

FASTA Seq from: 1 to: 1398 June 18, 1995 21:19

The best scores are:	opt*
Rahnella aquatilis (2-87)	5316
Yersinia pseudotuberculosis ...	5206
Hafnia alvei 16S ribosomal RNA.	5177
Y. intermedia (ER-3854) gene 16S ...	5165
Y. pestis 16S ribosomal RNA...	5163
Y. kristensenii (ER-2812) gene ...	5142
Y. rohdei (ER-2935) gene 16S rib...	5123
Y. mollaretii (ER-2975) gene 16...	5118
Y. enterocolitica 16S ribos...	5117
Erwinia carotovora 16S ribosomal ...	5114
Serratia marcescens 16S ribosomal…	5063
Y.bercovieri (ER-2937) gene 16...	5057
Glossina pallidipes (tsetse) endo...	4977
Plesiomonas shigelloides 16S rDNA...	4858
E. coli genomic sequence ...	4953
Proteus vulgaris 16S rRNA gene.	4934
Sitophilus zeamais 16S.Endosymbio...	4756
Arsenophonus nasoniae 16S ribosom...	4683
Photobacterium phosphoreum (ATCC ...	4678
Vibrio mimicus (ATCC 33653T) gene for ...	4664
V. cholerae (ATCC 14035T) gene for...	4652
A. shubertii (ATCC 43700T) gene fo...	4651
A. veronii (ATCC 35624T) gene for ...	4644
Y. frederiksenii (ER-1419) gene ...	4622
A. jandaei (ATCC 49568) gene for 1...	4618
Photobacterium leiognathi (ATCC 2...	4579
V. anguillarum 16S ribosomal ...	4488
Pasteurella testudinis 16S riboso...	4444
Barophilic bacteria gene fo 16S r...	4334
V. splendidus (SCB8) gene for 16S ...	4331
A. katoptron symbiont 16S rRNA gen...	4271
Buchnera aphidicola (primary endo...	4219
Kryptophanaron alfredi symbiont g...	4124
Halomonas meridiana DSM 5425) 16S...	4007
Halomonas subglaciescola DSM	3999
V. costicola (ATCC 35508T) gene fo...	3935
Sitophilus oryzae 16S rRNA ...	3772
Solemya reidi gill symbiont ribos...	2850

* The optimised (opt) score generated by FASTA represents the overall DNA identity of two sequences following joining of the best initial local alignments (Pearson & Lipman, 1988).

Figure 3. FASTA alignment between *E. americana* and *Rahnella aquatilis* (identical nucleotides boxed). *E. americana* (PI98 / *R. aquatilis* (2-87) 16S rDNA 97.1 % identity in 1399 bp overlap

```
Ewingella     1  --AGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGCAGCGGGAAGTAGCTTGCTACTTTGCCGGCGAGCGGCGGACGGG   104
Rahnella      1  AGAGTTTGATNATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGCAGCGNAAAGTAGCTTGCTACTTTGCCGGCGAGCGGCGGACGGG   106

Ewingella   105  TGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATGACCTCGAAAGAGCAAAGTGGGGGACCTTCGGGCCTCAC   210
Rahnella    107  TGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATGACCTCGAAAGAGCAAAGTGGGGGATCTTCGGACCTCAC   212

Ewingella   211  GCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGAAGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTG   316
Rahnella    213  GCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGA-GGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTG   317

Ewingella   317  AGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGC-TTCGGGTTGTA   421
Rahnella    318  AGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTAGGGTTGTA   423

Ewingella   422  AAGCACTTTCAGCGAGGAGGAAGGCATTAAGGTTAATAACCTTAGTGATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC   527
Rahnella    424  AAGCACTTTCAGCGAGGAGGAAGGCACCATACTTAATACGTGTGGTGATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC   529

Ewingella   528  GGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGAGCTTAACTTGGGAACTGCATTTGAAA   633
Rahnella    530  GGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGCGCTTAACGTGGGAACTGCATTTGAAA   635

Ewingella   634  CTGGCAAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGGCTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAG   739
Rahnella    636  CTGGCAAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATANNNNNNNNNAGGCGGCCCCCTGGACAAAG   741

Ewingella   740  ACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGTGGGCTTGACCCGTGGCT   845
Rahnella    742  ACTGACGCTCAGGTGCGAAANNGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCT   847

Ewingella   846  TCCGGACGTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAAT   951
Rahnella    848  TCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAAT   953

Ewingella   952  TCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAATTCGCTAGAGATAGCTTAGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTC   1057
Rahnella    954  TCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCACGGAATTCGCCAGAGATGGCTTAGTGCCTTCGGGAACCGTGAGACAGGTGCTGCATGGCTGTCGTC   1059

Ewingella  1058  AGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCACGTCATGGTGGGAACTCAAAGGAGACTGCCGGTGATAAA   1163
Rahnella   1060  AGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCACGTAATGGTGGGAACTCAAAGGAGACTGCCGGTGATAAA   1165

Ewingella  1164  CCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCATATACAAAGAGAAGCGAACTCGCGAGAGCAAGC   1269
Rahnella   1166  CCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCATATACAAAGAGAAGCGAACTCGCGAGAGCAAGC   1271

Ewingella  1270  GGACCTCATAAAGTATGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATGAGAATGCTACGGTGAATACGTTCC   1375
Rahnella   1272  GGACCTCATAAAGTATGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCC   1377

Ewingella  1376  CGGGCCTTGCACACACCGCCCGT   1398
Rahnella   1378  CGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACTTCGGGAGGGCGCTTACCACTTTGTGATTCATGACTGGGGT   1483

Rahnella   1484  GAAGTCGTAACAAGGTAACCGTAGGGGAACTGCGGTTGGATCACCTCCTTA   1534
```

Other significant matches were with various members of the *Enterobacteriaceae*, notably several members of the genus *Yersinia*. Also giving high scores were *Hafnia alvei*, *Erwinia carotovora* and several *Vibrio* species (the *Vibrionaceae* are a closely allied group to the *Enterobacteriaceae*). Other matches, for example, with *Arsenophonus* sp., were with other members within the Gamma subclass of the *Proteobacteria*.

Genomic DNA Hybridisation analysis

DNA hybridisation (Figure 4, Table 5) effectively identified ISN strains as *E. americana.*

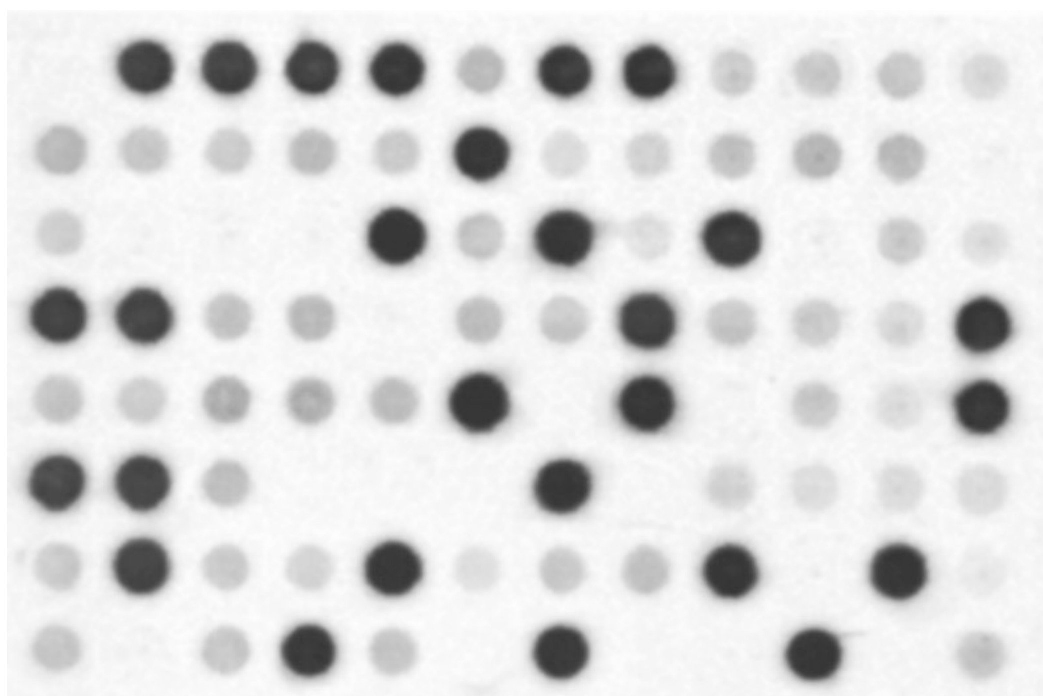


Figure 4. Hybridisation filter containing dot-blotted genomic DNAs (randomised arrangement) from ISN strains as well as reference strains (for composition, see table 5) probed with ISN strain PI98 genomic DNA. Depicted example was exposed to X-ray film (Kodak Biomax MR) following chemiluminescent detection (stringency wash 68˚C, 0.1 x SSC, 1% SDS). The darkest spots on this image are all occupied by either DNA from ISN strains or *E. americana* (NCTC 12157). Blank areas were control spots, thereby allowing calibration of background readings. Intermediate areas are occupied by DNAs from species taxonomically related to *E. americana*. Signals were subsequently quantified directly from the hybridised filter by a computerised phosphor imager (Bio-Rad model GS363).

Relative binding ratios (RBR) between strain PI98 and other ISN strains were all above 80% (Table 5), which is comfortably within the accepted species breakpoint of 70% (Wayne *et al*., 1987). The RBR with the type strain of *E. americana* was 88.8%, indicating species identity; and was in fact, less divergent than the values with some other ISN strains. The next most related species was *Rahnella aquatilis,* which was also the case with the 16S rDNA data.

Table 5. Reassociation of DNA from strain PI98 with that of other ISN and reference (type) strains at 68˚C and 0.1xSSC. Hybridisation signals measured in a phosphor imager with chemiluminescent cassette. 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 ± 8.5
ISN Strain PI180 (NCPPB 3908)	91.8 ± 9.2
ISN Strain PI200 (NCPPB 3909)	88.7 ± 6.7
ISN Strain PI74 (NCPPB 3903)	88.2 ± 7.1
ISN Strain PI145 (NCPPB 3906)	87.6 ± 7.4
ISN Strain PI166 (NCPPB 3907)	85.1 ± 1.0
ISN Strain PI91 (NCPPB 3904)	80.1 ± 1.7
Ewingella americana NCTC 12157	88.8 ± 6.3
Rahnella aquatilis 21E	26.8 ± 3.6
Erwinia amyluria NCPPB 683	22.6 ± 2.0
Hafnia alvei NCIMB 11999	22.0 ± 2.6
Enterobacter cloacae NCIMB 10101	21.9 ±2.2
Cedecea davisae NCIMB 11889	21.1 ± 0.7
Erwinia salicis NCPPB 447	20.8 ± 2.0
Erwinia carotovora subsp. carotovora NCPPB 312	20.6 ± 1.2
Pantoea punctata NCPPB 3820	20.3 ± 2.1
Pantoea citrea NCPPB 3826	20.1 ± 1.7
Proteus vulgaris NCIMB 4175	18.0 ± 1.2
Erwinia cypripedii NCPPB 3004	16.4 ± 2.2
E. coli K12 / DH5a	12.3 ± 0.2

DISCUSSION

Conventional phenotypic examination of ISN strains indicated that this group was typical of the *Enterobacteriaceae*; that is, the bacteria were facultatively anaerobic Gram-negative straight rods. The strains also share several features in common with certain members of the plant pathogenic genus *Erwinia* (Verdonk *et al.,* 1987). Poppe (1978), in a review of mushroom domestication and disease, mentions an unspecified disease associated with an *Erwinia* sp. and it is possible that the ISN-type bacteria could have been interpreted as such. Biochemical profiling, however, provided powerful evidence that the ISN strains were closely related to the unusual species, *Ewingella americana*. This species was formally known only from clinical specimens, and appeared to be an opportunistic human pathogen (Pien *et al.,* 1983; Devreese *et al.,* 1992). The environmental reservoir, or primary host of this organism was also unknown. As discussed before, ISN strains were chitinolytic and positive for Tween 80 esterase. Both these characters were recorded as negative in the original and subsequent descriptions of the species, but in tests adopted here, both characters were found to be positive. The reason for the discrepancy with Tween 80 is possibly due to degradation of the lipid during sterilisation to smaller moieties susceptible to the bacterial lipase, since *E. americana* is recorded as being positive for Tween 20 and Tween 40 utilisation. In the case of chitinase, the discrepancy is likely to be due to the fact that bacteria were formally screened by simple growth tests. Both ISN strains and *E. americana* NCTC 12157 failed to grow on minimal medium supplemented with colloidal chitin and glycol chitin (Chapter 5). However, all ISN strains grew and rapidly produced clearing zones on LB agar supplemented with RBV stained CM-chitin. This test does not rely on the bacteria being able to utilise the products of chitin digestion, but simply demonstrates the presence of chitinase. It is likely to be the case that the bacteria are able to digest the

chitin polymer into chitiooligosaccharides, but are unable to digest these products to the monomer, N-acetylglucosamine, which these strains can utilise as a carbon source for growth.

The 16S rDNA data again indicated ISN strain PI98 to be a member of the *Enterobacteriaceae*. The closest match was with *Rahnella aquatilis* to which *E. americana* is phenotypically related. The alignment displays a non-random arrangement of variable regions which is thought to be due to the distribution of non-lethal sites on the 16S rRNA molecule (Gutell *et al*., 1994). A match of 97.1% probably does not indicate a species identity however. This is especially true of the *Enterobacteriaceae* which are a highly homogenous group at the rDNA level (Gross & Holmes, 1990; Young, 1994). The databases of 16S rDNA sequences, however, are not complete and *Enterobacteriaceae* are somewhat underrepresented, given their familiarity and general microbiological importance. The 16S rRNA gene of *E. americana* has not previously appeared in the databases and so a direct comparison was unfortunately impossible.

DNA hybridisation clearly identified the ISN strains as belonging to *E. americana*. The relative binding ratio between the type strain NCTC 12157 and ISN strain PI98 was 88.8%, which was greater than the values from ISN strains PI91, PI166 and PI145. In the original description of *E. americana* (Grimont *et al.,* 1983), the range of relative binding ratios within the collection of clinical isolates using the S1 nuclease method was 73-107% at 60˚C and 73-104% at 75˚C, with most strains measuring above 80% at both stringencies. This range is consistent with the results obtained here, using a modified chemiluminescent filter binding method. As in this study, *Rahnella aquatilis* was the most homologous species to *E. americana* in the original report, having a RBR of 21%. A RBR of 26.8 ± 3.6% was obtained here, which is somewhat higher than the value obtained with the S1 method. This difference was repeated with other species, meaning that the method implemented

here is somewhat less stringent at lower homology levels, although still yields similar inferences. The results reported here were produced following stringency washing of filters at 68˚C. Washes at 75˚C were found to reduce the signals from non-homologous species while maintaining the high readings from wells with *E. americana* or ISN strain DNA (data not shown), but required long exposure times to yield significant readings above background. This chemiluminescent method demonstrates considerable potential to replace hazardous, costly and time-consuming radioactive methods for DNA reassociation analysis.

The proof that mushroom internal stipe necrosis is caused by *Ewingella americana* is significant in that it is the second mushroom disease to be shown to be caused by a bacterium formally thought to be a human pathogen. The other example is cavity disease caused by *Pseudomonas* (now *Burkholderia*) *cepacia* (Gill & Cole, 1992) which, as well as being isolated from soil and plant sources, has also been associated with lung disease in humans (Goldmann & Klinger, 1986). However, the identification of the causative agent was based solely on biochemical tests and probably requires confirmation by DNA reassociation analysis.

In this study, API tests (both API 20E and API 50CHE) were more successful than the automated FAME test in identifying the ISN strains as *E. americana.* This was entirely due to the relative completeness of the libraries concerned. The API systems are geared more strongly towards bacteria of medical importance, but are particularly weak for plant pathogens and environmental isolates. API tests appear to identify *E. americana* reliably and have the advantages of rapidity (48 h) and economy. FAME analysis, however, requires that the strain is sent to a reference laboratory, with the obvious concomitant delays. An effective screening test for mushrooms affected with internal stipe necrosis is to sample stipes bacteriologically on CM-chitin-RBV agar incubated anaerobically. After 48 h, chitinolytic colonies can then be subcultured aerobically and facultatively anaerobic

colonies then analysed by API 20E strips. This procedure should routinely select and identify *E. americana* in diseased mushrooms, which may later be confirmed by API 50CHE tests, or DNA hybridisation.

CHAPTER 5
Purification and some properties of a chitinase from *Ewingella americana*

INTRODUCTION

The possible significance of chitinase production in *E. americana* pathogenicity towards *A. bisporus* was discussed in Chapter 1. The work described in the next two Chapters attempts to characterise the chitinolytic activity of the ISN causative agent, both biochemically and genetically. Such data may then be directed towards the confirmation of chitinase as a virulence factor in ISN pathogenesis.

Chitin is the second most abundant structural polymer in nature after cellulose, and as such, constitutes an important renewable resource (Shaikh & Deshpande, 1993). As well as its role in the cell walls of many fungi (Cabib, 1987), chitin is also found in abundance in arthropods and is also present in certain algae and protozoa. Reports of the presence of chitin in primitive chordates have been contradictory and may have been due to contamination of specimens with chitin of planktonic origin (Rudall & Kenchington, 1973).

Chitin is an unbranched polymer of β-1,4-linked *N*-acetylglucosamine residues (GlcNAc; 2-acetamido-2-deoxy-D-glucose; NAG). The linear chitin chains associate by strong hydrogen bonding via the >N-H groups of one chain and the >C=O groups of the second, thereby forming an antiparallel structure (α chitin), eventually leading to fibril formation (Minke & Blackwell, 1978). The partially deacetylated derivative of chitin is called chitosan, forming a glucosamine polymer. In nature, this polymer is restricted to zygomycetous fungi.

Enzymatic degradation of chitin

The complete degradation of chitin chains to their component *N*-acetylglucosamine units is often achieved by a complex of enzymes acting to initially break the chains into short, random chitooligosaccharides. Separate enzymes then act to break these oligomers down into their component monomers. This chitinolytic system has been found in both organisms that themselves contain chitin, production of which is at least partially related to growth, and in other organisms, such as bacteria, plants and animals, in which the enzymes probably have nutritional, defensive or other ecological roles (Flach *et al.,* 1992).

Current enzyme nomenclature recognises two main classes of chitinolytic enzyme: (1) endochitinase (EC 3.2.1.14) and (2) *N*-acetylglucosaminidase (EC 3.2.1.30; equivalent to the now disused chitobiase). Endochitinases catalyse the random cleavage of β-1,4 linkages at internal points in the chitin chains. *N*-acetylglucosaminidases, on the other hand, mediates the cleavage of terminal NAG units from the non-reducing end of the chitin chains. Evidence for the former mode of activity has been provided by chromatographic analysis of the products of chitin digestion, where randomly sized chitooligosaccharides are detected. The ultimate product from such activity is usually a predominance of the dimer, chitobiose. Attempts have also been made to infer this type of activity by viscosimetry, where a rapid reduction in the viscosity of a soluble chitin preparation is observed (Ohtakara, 1988; Vyas & Deshpande, 1993). A variant of this type of chitinase is found in enzymes that appear to catalyse the stepwise release of chitobiose units from the chitin chains. Such enzymes have been referred to as exochitinases, or chitobiosidases (Sahai & Manocha, 1993; Tronsmo & Harman, 1993), but are not currently recognised by *Enzyme Nomenclature*. Recently, the mode of action of chitinases has been inferred by a number of workers, by observation of activity against substituted chitooligosaccharides (McCreath & Gooday, 1992; Tronsmo &

Harman, 1993; Lorito *et al.*, 1993b; Frändberg & Schnürer, 1994). These substrates have one of their constitutive NAG units replaced by either a chromogenic molecule, such as *p*-nitrophenol, or a fluorogenic molecule, such as 4-methylumbelliferone. Hydrolysis of the substituted chitooligosaccharide results in the release and activation of the reporter molecule (Figure 1).

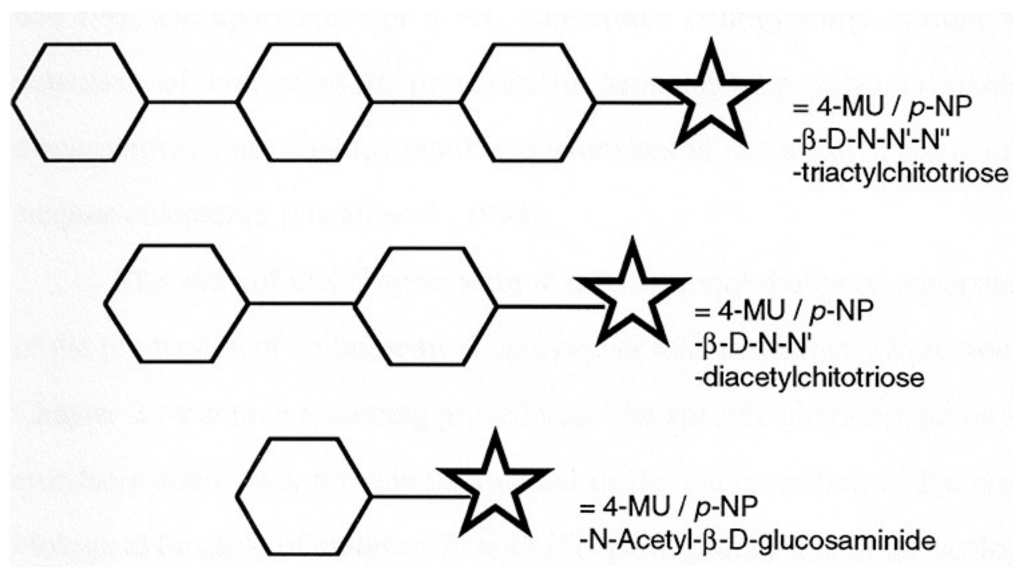


Figure 1. Representation of chromogenic / fluorogenic derivatives of chitooligosaccharides. n= a *p*-nitrophenol or 4-methylumbelliferone group, substituting a GlcNAc residue, β-1,4 linked. (Adapted from Haran *et al.*, 1995).

N-acetylglucosaminidases activate all three types of derivatised substrate due to the stepwise release of NAG units, eventually liberating the reporter molecule. Chitinases, however, have no activity against the substituted dimer, but may randomly release reporter from both the trimer and tetramer. Quantitatively in this case, it is uncertain as to the proportion of molecules cleaved at the terminal reporter linkage, or at other β-1,4 linkages. Chitinases with exo-type action generally give a positive reaction only with the substituted trimer.

Many organisms have been demonstrated to produce a complex of chitinolytic enzymes. *Serratia marcescens* strain QMB1466, for example, was

shown to produce 5 different chitinolytic enzymes on induction by chitin (Fuchs *et al.,* 1986). Such a multiplicity of chitinases, however, may be the result of differential processing of a precursor, as is the case in *Streptomyces olivaceoviridis* (Romageura *et al*., 1992). There is therefore a definite requirement that such enzymes be in pure form, when attempts are made to fully characterise chitinolytic activity. For more qualitative experiments, however, the application of 4-MU substituted chitooligosaccharides to the detection of chitinases in preparations separated by polyacrylamide gel electrophoresis has enabled rapid improvements in our understanding of such enzyme complexes (Haran *et al*., 1995).

The aims of this chapter were to effect a more thorough understanding of the production of chitinase by *E. americana* than that achieved previously in Chapter 2 by simple screening procedures. The specific characterisation of the enzyme/s could then provide data useful in the interpretation of the possible biological function of chitinase in both ISN pathogenesis and in the ecology of *E. americana*.

MATERIALS AND METHODS

Preparation of chitin derivatives:

Ball milled native chitin

Technical grade crab shell chitin (100 g; Sigma) was powdered by milling for 72 h in a Pascall ball mill at 60 rpm. The material so formed was passed through a flour sieve and stored in an airtight container at room temperature.

Colloidal (swollen) chitin

Colloidal chitin was prepared by an adaption of the method of Shimahara & Takiguchi (1988). Ball milled chitin (20 g) was gradually added to 500 ml concentrated HCl while stirring at 4˚C, and allowed to dissolve over 3 h. Insoluble materials were removed by filtration through glass wool, and the demineralised and purified chitin precipitated by the addition of 500 ml 50% (w/v) ethanol. The

mixture was stirred for 3 h and the precipitate collected by filtration through a sintered glass filter. The colloidal chitin was then washed with distilled water until the pH reached neutrality and collected by centrifugation. For binding applications, the product was kept in the hydrated form at 4˚C; or for long-term storage, lyophilised and placed at -20˚C.

Glycol chitin

A solution of glycol chitin was prepared according to Trudel & Asselin (1989). Glycol chitosan (1 g; Sigma) was dissolved in 20 ml of 10% (v/v) acetic acid, mixed with 30 ml methanol and filtered using glass wool. Acetic anhydride (7.5 ml) was added to the filtrate, and the resultant gel allowed to stand at room temperature for 30 min, after which, the gel was homogenised in a Waring blender. One volume of methanol was added and the precipitate collected by centrifugation. The pellet was washed several times with distilled water, and the product resuspended in 100 ml 0.05 M sodium acetate buffer pH 5.5. Sodium azide (0.02% w/v) was added as a preservative and the product stored at 4˚C.

Carboxymethylated (CM) chitin

Alkaline, or carboxymethylated chitin (Muzzarelli, 1988) was prepared according to Trujillo (1968) as modified by Wirth & Wolf (1990). Ball milled chitin (10 g) was added with constant stirring to 125 ml 65% (w/v) NaOH. Stirring was continued for 1 h at room temperature, after which the insoluble products were collected with a sintered glass filter. Proteins and lipids were removed by stirring the chitin for 1 h in a solution of 15 g chloroacetic acid in propan-2-ol (125 ml). Insoluble products were again collected by filtration, and washed with 125 ml propan-2-ol. The CM chitin was then dissolved over 24 h in 1 l distilled water with constant stirring and the pH adjusted to 7.0 with HCl. Dissolution was assisted by homogenisation in a Waring blender. Yield was measured gravimetrically following lyophilisation of a 5 ml sample. CM-Chitin was autoclaved and stored at -20˚C.

Dye labelling of CM chitin

The methods of Wirth & Wolf (1990) were used to covalently link the dye, Remazol brilliant violet 5R (RBV; Sigma) to chitin derivatives. RBV (5 g) was added at 50˚C to 1 l of the CM chitin prepared as above. While stirring constantly for 45 min, 100 g Na_2SO_4 was gradually added. A 50 ml solution of $Na_3PO_4.12H_20$ (7.84 g) was then added and stirring continued for 75 min at 50˚C. Unlinked dye and salts were then removed by dialysis against demineralised H_2O and any insoluble debris removed by filtration through glass wool. The product was then autoclaved and stored at -20˚C.

Plate screens for chitinase induction

LB agar was supplemented **with** 2.5 mg ml^{-1} CM-Chitin-RBV and also with 10 g l^{-1} glucose or 10 g l^{-1} N-acetylglucosamine. ISN strains of *E. americana* were plated on this medium and incubated aerobically for 48 h at 28˚C. Chitinolytic clearing zones were then observed with bacteria grown in the presence of the above sugars.

Plate test for lysozyme activity

LB agar was supplemented with 0.2% (w/v) *Micrococcus lysodeikticus* (*luteus*) cells (Sigma), autoclaved at 121˚C for 15 min and plates poured. Bacteria were then plated on this medium, incubated as above. Lysozyme activity was indicated by production of clearing zones in the otherwise opaque medium.

Preparation of crude bacterial protein:

Strains and fermentation

For purification studies, strain PI98 was grown in a total culture volume of 3 l in a defined broth; l^{-1}: lactose 2 g, mannitol 10 g, K_2HPO_4 7 g, KH_2PO_4 2 g, ammonium sulphate 0.5 g, 0.1 ml vitamin cocktail (l^{-1}: biotin 2 mg; pyridoxine 0.5 g; aneurine 0.5 g; nicotinic acid 1 g; riboflavine 1 g); and incubated at 28˚C for 72 h in shake flasks. Subsequently, cells were removed by centrifugation at 17,000 g and pH adjusted to 5.5. The culture supernatant was then passed through a sterile 0.45 µm

filter and sodium azide (0.02% w/v) added as preservative. A 100 ml batch of the above medium was also prepared containing 1 ml of the glycol chitin solution. The inoculated flask containing this medium was incubated as above and subsequently assayed for chitinase. Crude enzyme was produced in 72 h cultures in LB broth. Cultures were centrifuged at 17,000 g and the supernatant passed through a sterile 0.45 µm pore filter. These cell-free filtrates were then stored at -20˚C until needed. A plate screening test was also set up, whereby 5 ml l^{-1} glycol chitin solution was added to the minimal medium utilised for carbon source utilisation tests, as used in Chapter 2. Following streaking of ISN strains on this medium, plates were incubated for 7 days at 28˚C, after which, assessment was made as to the growth of *E. americana* with chitin as sole source of carbon.

Partial purification of chitin binding fractions by batch affinity chromatography

Attempts were made to purify *E. americana* chitinase using a method adapted from Deane (1994). The culture supernatant prepared as above was cooled to 4˚C and 10 g l^{-1} (pressed wet weight) colloidal chitin added. The mixture was gently stirred using a magnetic stirrer for 2 h, after which, the chitin was collected by centrifugation. Non-specifically bound proteins were removed by 2 washes with 0.5 M NaCl in 50 mM sodium acetate buffer, pH 5.5. Binding fractions were eluted by 2 washes with 200 mM ammonium acetate buffer, pH 9.0. The product was then passed through a 0.45 µm filter, dialysed against distilled water and concentrated by freeze drying.

Hydrophobic interaction chromatography (HIC)

A sample of culture supernatant was adjusted to 0.5 M ammonium sulphate and pH 7.0, and applied to a 15 ml column of phenyl sepharose CL-6B (Pharmacia), while constantly monitoring at 280 nm. Maximum flow rates were adjusted according to chromatography resin manufacturer's instructions. The void from the column was collected, and the column washed with 0.1 M ammonium sulphate, pH 7.0. Bound proteins were then eluted with a 100 ml linear gradient of 0-30% (v/v) propan-2-ol and peak fractions collected. The fraction containing chitinase activity was then freeze dried and stored at -20˚C.

Protein quantification

Solutions were assayed for protein content using an assay kit (Bio-Rad) based on the dye binding method of Bradford (1976). A standard curve was constructed using bovine serum albumin (2.5-25 µg ml^{-1}) as the reference protein according to manufacturer's instructions.

Chitinase assay

CM-chitin-RBV (5.0 mg ml^{-1} sterile stock solution) was used as substrate in chitinase assays to define activity under a range of varying conditions. This method was chosen because of its sensitivity and utility in cases where the components of the chitinolytic system are unknown (Wirth & Wolf, 1990). Furthermore, the assay was found to be specific for chitinase activity; in particular, for endo-type enzymes. The assay was also unaffected by contaminating *N*-acetyl-β-D-glucosaminidase, β-glucosidase, β-galactosidase and *N*-acetyl-muraminidase (Lysozyme) activities (Saborowski *et al.,* 1993). Assays were performed in sterile 1.5 ml reaction tubes (Eppendorf). The standard reaction mixture consisted of 400 µl buffer (0.2 M phosphate, pH 6.5), 200 µl CM-Chitin-RBV solution and 200 µl enzyme solution. All solutions were kept on ice prior to mixing and incubation at 37˚C for an appropriate time, usually 10 min. Reactions were then returned to ice, and 200 µl

1M HCl added. Tubes were then incubated on ice for a further 15 min to effect complete acid precipitation of undigested CM-Chitin. Tubes were then centrifuged, and absorbance read at 550 nm, following blanking against a control reaction where acid was added simultaneously with the other assay components.

Activity of chitinase against chromogenic soluble chitin derivatives

The soluble chitin derivatives *p*-nitrophenyl-*N*-acetyl β-D-glucosaminide [pNP-GlcNAc], *p*-nitrophenyl-β-D-*N,N'*-diacetylchitobiose [pNP-$(GlcNAc)_2$] and *p*-nitrophenyl-β-D-*N,N',N"*-triacetylchitotriose [pNP-$(GlcNAc)_3$] (Sigma) were used as a chromogenic assay for *E. americana* chitinase (Roberts and Selitrennikoff, 1988). Activity against these substrates was used to infer the mode of action of the enzyme (Lorito *et al.*, 1993b; Chernin *et al.*, 1995). A 125 µM solution of each derivative was prepared in 50mM phosphate buffer pH 6.5. Substrate (900 µl) was dispensed in an Eppendorf tube, and preincubated at 37˚C for 5 min, after which 100 ng enzyme was added. Tubes were incubated for 20 min, after which, reactions were stopped by the addition of 100 µl 1 M NaOH. The released *p*-nitrophenol was estimated against a previously constructed standard curve (in the range 0.1-50 µmol) by measuring absorbance at 405 nm.

Polyacrylamide gel electrophoresis under denaturing conditions (SDS -PAGE)

Protein preparations were resolved using the discontinuous buffer system of Laemmli (1970), using a Protein II xi dual slab cell apparatus (Bio-Rad). Gels were prepared using Protogel (National Diagnostics) acrylamide solution (acrylamide: bisacrylamide ratio 30:0.8).

Prior to loading, protein samples were mixed 1:0.5 with denaturing loading buffer (0.2 M Tris-HCl pH 6.8, 6% (w/v) SDS, 130 mM dithiothreitol, 10% (v/v) glycerol, 0.03% (w/v) phenol red) and boiled for 5 min. Molecular weight markers were: Glutamic dehydrogenase (62 kDa), Aldolase (47.5 kDa), Triosephosphate isomerase (32.5 kDa), β-Lactoglobulin A (25 kDa), Lysozyme (16.5 kDa),

Aprotinin (6.5 kDa). Gels were electrophoresed at 4˚C at 200 V in Tris-glycine-SDS running buffer, until the tracking dye neared the bottom of the gel, typically following 5 h. Following electrophoresis, gels were fixed in a solution of 40% (v/v) methanol and 10% (v/v) acetic acid and stained with Coomassie blue. In the case of the chitinase purified from *E. americana*, it was found necessary to add urea to 3 M prior to the loading buffer for Laemmli SDS-PAGE gels, in order to preserve protein solubility.

Detection of chitinolytic activity in polyacrylamide gels

Chitinolytic activity was detected in polyacrylamide gels according to Tronsmo & Harman (1993) and Chernin *et al.* (1995). The soluble fluorogenic derivative of chitin, 4-methylumbelliferyl-β–D-*N,N′,N″*-triacetylchitotriose [4-MU-$(GlcNAc)_3$] (Sigma) was used as a specific activity stain following electrophoresis and removal of SDS with a 30 min wash with a 5% (v/v) Triton X-100 solution. Additionally, glycol chitin was incorporated in some gels and stained after protein renaturation with the chitin binding fluorogen, calcofluor white (Trudel & Asselin, 1989). In both cases, gels were observed on a shortwave UV transilluminator.

Characterisation of E. americana chitinase activity:

Determination of temperature optimum for chitinase activity

The optimal temperature for *E. americana* PI98 chitinase activity was determined at pH 6.5 using the CM-chitin-RBV assay (Saborowski *et al.,* 1993). Reaction mixes were identical to the standard Eppendorf scale assay, but were preincubated at the required temperature for 10 min prior to adding 200 µl of crude cell-free filtrate obtained from a 72 h *E. americana* culture in LB broth. Activity was measured at temperatures between 25˚C-90˚C at increments of 5˚C.

Determination of thermal stability of chitinase

The temperature stability of chitinase was determined at pH 6.5 by preincubating enzyme solutions at temperatures between 25˚C-70˚C for 1 h. Thereafter, the standard chitinase assay was performed on the preheated samples.

Determination of optimal pH for chitinase activity

The effect of pH on *E. americana* chitinase was determined by varying the composition of the standard assay buffer between pH values 2.5-10.5 (citrate-phosphate-buffer, 0.2 M, pH 2.5-6.5; phosphate buffer, 0.2 M pH 6.5-8.5; glycine-NaOH, 0.2 M, pH 8.5-10.5) in increments of 0.5 pH units.

Determination of pH stability of chitinase

The effect of pH on the stability of chitinase was investigated by incubating 200 µl enzyme solutions for 1 h at various pH values (Using 50 µl of the above 0.2 M buffers) at 37˚C. Thereafter, the treated enzyme solutions were adjusted to pH 6.5 by addition of 200 µl 0.5 M phosphate buffer and the standard assay set up to measure residual activity.

Effect of ionic strength on chitinase activity

The effect of ionic strength on chitinase activity was investigated by the addition of NaCl to the standard assay mixture at pH 6.5 and at 37˚C. Salt concentrations were tested between 0.1-4.5 M.

Effect of metal ions on chitinase activity

The influence of several metal ions on the activity of chitinase was investigated. Metal salts at 20 mM were added to the standard assay mix at pH 6.5 and at 37˚C. Metal ions tested were: K^{+}, Mg^{2+}, Ca^{2+}, Na+, Mn^{2+}, Zn^{2+}, Fe^{2+}, Hg^{2+}, Co^{2+} and Cu^{2+} .

Effect of EDTA, SDS and urea on chitinase activity

The possible inhibitory effect of EDTA, SDS and Urea (all Sigma) on chitinase activity was investigated by addition of the above chemicals at both 10 mM and 20 mM to the standard assay mix.

RESULTS

Regulation of chitinase and lysozyme activity

Chitinolytic clearing zones were produced by *E. americana* strains plated in the presence of both glucose and N-acetylglucosamine, indicating that production of this enzyme was not repressed by these treatments. Furthermore, chitinase was found to be produced by liquid cultures of *E. americana* in similar concentrations both in the presence and absence of possibly inducing glycol chitin. This indicates that production of chitinase in *E. americana* is constitutive. Interestingly, ISN strains failed to grow with chitin as the sole source of carbon. Clearing zones were not observed on media containing *Micrococcus lysodiekticus* cell walls, indicating absence of lysozyme activity in *E. americana* and in its chitinase.

Chitinase purification

Affinity chromatography failed to adequately purify the chitinase of *E. americana*. Yields of activity recovered were very low, and Coomassie stained SDS-PAGE gels of the released chitin binding products displayed several contaminating bands (data not shown). The major product isolated by affinity chromatography was a protein with very low solubility in water, which could be dissolved in 15% (v/v) acetic acid. This material had no activity against CM-chitin-RBV, but could represent a specific chitin-binding protein in *E. americana*.

Passage of culture filtrates through the phenyl sepharose column at the indicated ammonium sulphate concentration, extracted virtually all of the chitinase activity as measured by CM-chitin-RBV hydrolysis. This activity was retained on

the column during the washing phase. Elution of activity occurred when this buffer was replaced by distilled water. A minor peak of activity was also produced when the column was washed with up to 30% (v/v) isopropanol. The major protein peak released by 30% (v/v) isopropanol appeared similar to the major protein obtained by affinity chromatography.

Analysis of the chitinolytic fraction following concentration by reverse dialysis against PEG8000 (Sigma), revealed a single band in SDS-PAGE gels stained with Coomassie blue (Figure 1). It was found necessary to include 30% (w/v) urea along with reducing gel loading buffer (NEB) to preserve solubility of samples prior to boiling. This treatment was also found necessary in order to achieve consistent migration of the chitinase in SDS-PAGE.

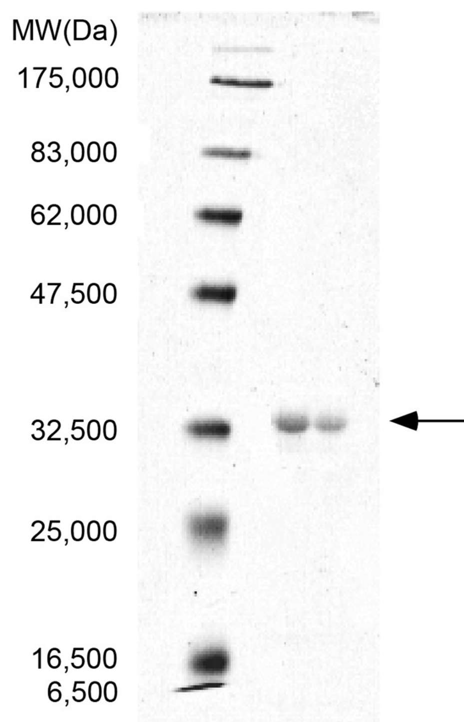


Figure 1. Chitinase purified by HIC, from *E. americana* strain PI98 (arrowed, band appears double due to well damage), resolved on a 12% SDS-PAGE gel, and stained with Coomassie brilliant blue. Molecular weights are indicated by wide-range pre-stained markers (New England Biolabs).

Classification of chitinase activity

The purified *E. americana* chitinase was classified as an endochitinase by virtue of its similar activity against trimeric and tetrameric chitooligosaccharide derivatives. Little activity was observed against the dimeric derivative (Table 1).

Determination of multiplicity of chitinolytic enzymes in E. americana.

Activity gels (Figure 2) indicated that a single chitinase was probably present in crude culture filtrates, as detected by staining with fluorogenic derivative, 4-methylumbelliferyl-β–D-*N,N',N''*-triacetylchitotriose.

Table 1. Activity of purified *E. americana* strain PI98 chitinase against *p*-nitrophenyl derivatives of chitooligosaccharides (see methods section for details).

Substrate	Enzyme activity (μmol pNP μg^{-1} protein h^{-1})*
p-NP-NAG	0.7 ± 0.04
p-NP-chitiobiose	47.7 ± 6.4
p-NP-chitiotriose	49.2 ± 4.7

* Results are the mean of triplicate determinations ± SEM.

The active band migrated with the bromophenol blue marker dye in both native PAGE and SDS-PAGE, suggesting a much smaller molecular weight than the purification data would imply. However, Coomassie blue stained gels of purified chitinase run without vigorous pre-treatment of the protein with boiling SDS, β-mercaptoethanol and urea, also gave such an anomalous migration pattern. Molecular weight determination of poorly reduced chitinase by activity staining was therefore considered unreliable. Activity gels consisting of glycol chitin incorporated into an SDS-PAGE matrix were unsatisfactory. The enzyme appeared to bind to the substrate, even in the presence of SDS, forming a smear of activity at the top of the separating gel.

Figure 2. Chitinase activity detected by [4-MU-$(GlcNAc)_3$] in a 12% SDS-PAGE gel (Arrowed, band runs just behind the tracking dye in this preparation). Sample was a crude $(NH_4)_2SO_4$ precipitate (dialysed) from *E. americana* strain PI98 culture filtrate, prepared from a 48 h culture in LB broth. Prior to loading, proteins were mixed 2:1 with gel loading buffer (NEB), excluding reducing agent. Molecular weight markers were loaded and run in an ajacent well. No fluorescence was observed in the marker lane following activity staining.

Biochemical characterisation of E. americana PI98 chitinase

Since data from activity staining and purification work indicated that a single, endo-acting chitinase was probably present in *E. americana* culture filtrates, simply prepared crude enzyme preparations were used in subsequent characterisation assays.

The optimal temperature for the activity of *E. americana* PI98 chitinase was found to be 50˚C (Figure 3), and retained 80% of relative activity when preincubated at this temperature for 1 h (Figure 4). The enzyme was fully stable under the test conditions up to 40˚C, but rapidly lost activity at temperatures above 50˚C. The enzyme was virtually inactivated by preincubation at 70˚C.

The optimal pH for chitinase activity was found to be 6.5 (Figure 5). Activity was also significant at pH values below this, with 50% of activity being retained at pH 4.0. At alkaline pH's, however, activity was inhibited, dropping to below 20 % relative activity at pH 8.5. Acid pH favoured enzyme stability, and even at pH 6.5, the enzyme lost approximately 30% of its activity (Figure 6). The optimum reported here, therefore, may be affected by extended assay incubation periods. Greater than 50% of activity was lost by preincubating the enzyme at pH 8.5.

E. americana chitinase was not greatly affected by changes in the salt concentration of the incubation buffer (Figure 7). A NaCl concentration of 4 M was required to cause a 50% reduction in relative activity. Chitinase activity was also slightly favoured in buffers containing 1 M NaCl.

Urea, at the concentrations used, did not significantly affect chitinase activity and EDTA caused only a small reduction in activity (Table 2). SDS caused a reduction in activity of less than 50%, which correlates well with the observations of chitin binding and hydrolysis by the enzyme in SDS-PAGE gels containing glycol chitin. This indicates that *E. americana* chitinase is not fully denatured by SDS treatment alone.

Certain metal ions had a drastic effect on chitinase activity (Table 3). Cobalt and manganese, at 20 mM, almost abolished chitinase activity. Other divalent metal ions, with the exception of calcium and magnesium, also inhibited activity, but not to the extent of the above. Copper ions were found to affect the colour of the RBV stain used in the chitinase assay, and so could not be used in further tests.

Table 2. Effect of denaturants / inhibitors on chitinase activity.

Inhibitor	Relative activity *
none	100 ± 0.4
Urea, 20mM	95 ± 0.9
Urea, 10mM	96 ± 1.4
SDS, 20mM	53 ± 1.0
SDS, 10mM	55 ± 1.2
EDTA, 20mM	84 ± 1.2
EDTA, 10mM	88 ± 2.3

* Results are the mean of 3 determinations with (±) SEM indicated. Activities calculated as a percentage of the maximum which was taken as 100%.

Table 3. Effect of metal ions on the activity of *E. americana* PI98 chitinase.

Metal	Relative activity*
none	100 ± 0.9
Na^{+}	98 ± 1.8
K^{+}	90 ± 1.0
Mg^{2+}	97 ± 2.0
Ca^{2+}	92 ± 2.0
Zn^{2+}	47 ± 1.5
Fe^{2+}	31 ± 1.9
Hg^{2+}	25 ± 1.5
Mn^{2+}	4 ± 0.6
Co^{2+}	2 ± 0.7
Cu^{2+}	ND

*Results are the mean of triplicate tests, with ± SEM indicated. Activities calculated as a percentage of the control treatment which was taken as 100%. ND; not determined due to interference with the assay.

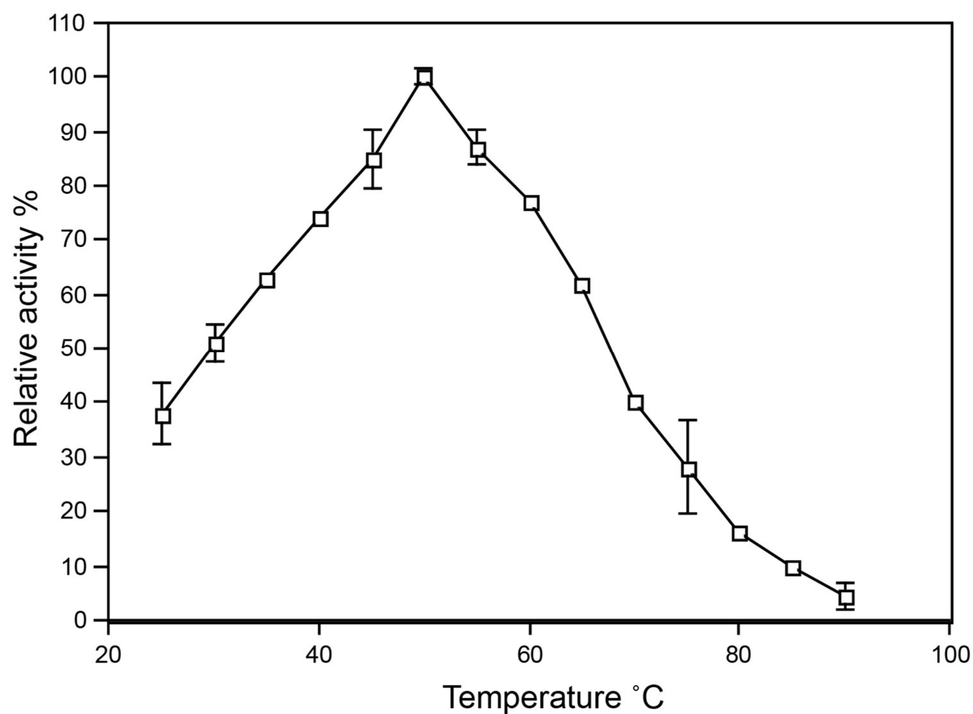


Figure 3. Effect of temperature on activity of *E. americana* (PI98) chitinase. Activities calculated as a percentage of the maximum which was taken as 100%. Results are the mean of 3 determinations, with SEM error bars indicated.

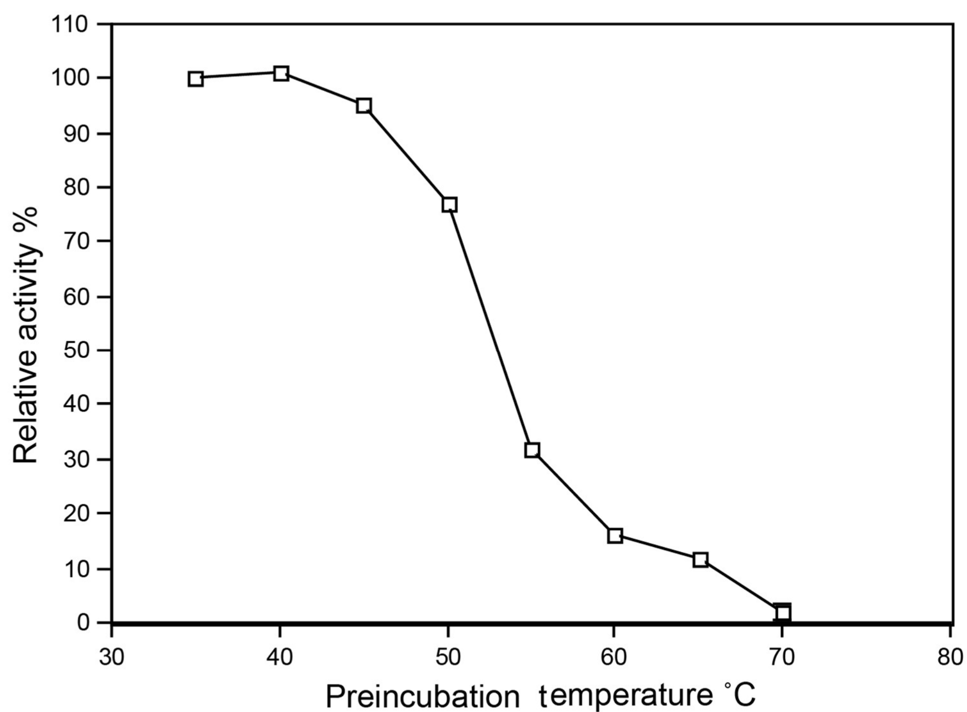


Figure 4. Thermal stability of *E. americana* (PI98) chitinase. Activities calculated as a percentage of the maximum which was taken as 100%. Results are the mean of 3 determinations, with SEM error bars indicated.

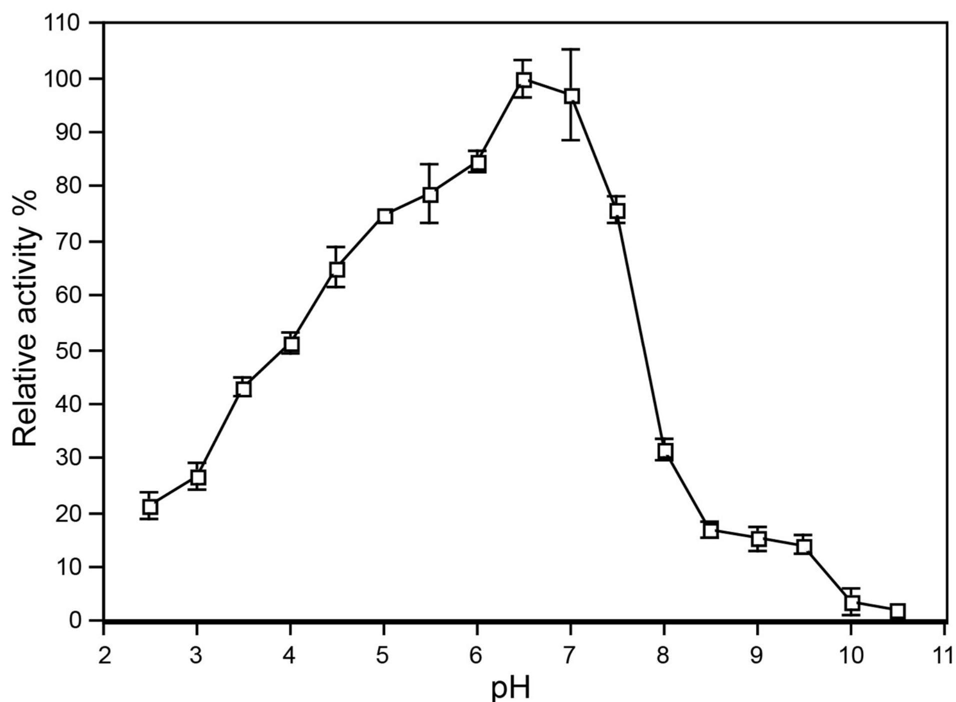


Figure 5. Effect of pH on the activity of *E. americana* (PI98) chitinase. Values from different overlapping buffer systems have been combined (See methods). Activities calculated as a percentage of the maximum which was taken as 100%. Results are the mean of 3 determinations, with SEM error bars indicated.

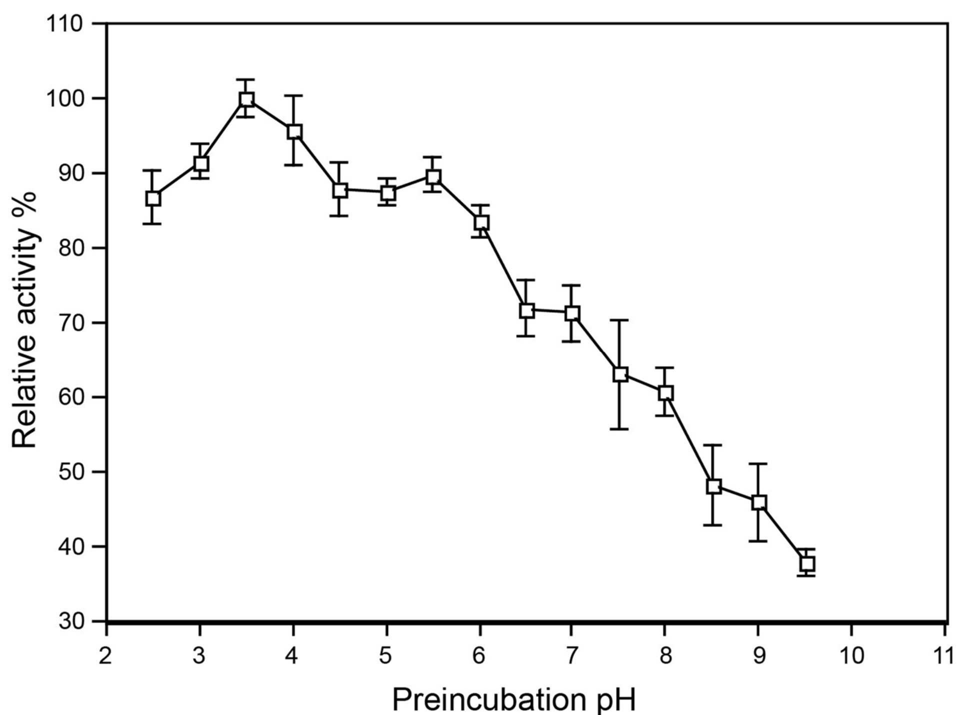


Figure 6. Effect of pH on the stability of *E. americana* (PI98) chitinase (See methods). Activities calculated as a percentage of the maximum which was taken as 100%. Results are the mean of 3 determinations, with SEM error bars indicated.

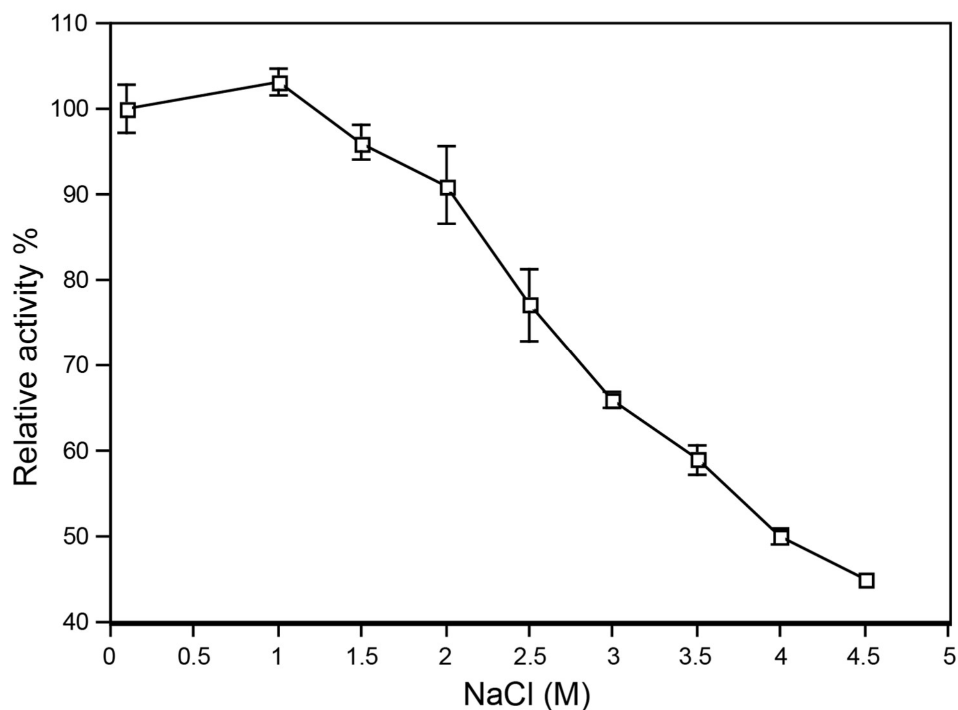


Figure 7. Effect of buffer salt concentration on activity of *E. americana* (PI98) chitinase. Activities calculated as a percentage of the maximum which was taken as 100%. Results are the mean of 3 determinations, with SEM error bars indicated.

DISCUSSION

Chitinase could be detected in LB broth cultures after overnight incubation, suggesting that it is secreted from *E. americana* cells. Furthermore, this production was not dependent upon the addition of chitin to the culture medium to effect induction. A possibility exists that the enzyme might be induced by substances present in the yeast extract of this medium, but fermentation in minimal media lacking chitin also resulted in chitinase production. Expression of the enzyme was not repressed by glucose or *N*-acetylglucosamine. We may conclude, therefore, that the production of this chitinase is constitutive in *E. americana*. Further work is necessary to compare the rates of chitinase production in different strains, since this could be significant in the light of the differences observed in the virulence of ISN

strains (Chapter 3). Additionally, chitinase production in relation to cellular growth phase, might provide valuable information on regulation.

A single chitinolytic band was detected in SDS-PAGE gels loaded with total protein from the *E. americana* culture filtrates, and this activity was characterised as of the endo-type. ISN strains failed to grow on minimal media with chitin as sole carbon source. This implies that these strains cannot grow on the products of chitin breakdown. These final products should be confirmed by chromatographic analysis, but it is likely that these consist largely of chitobiose, which *E. americana* may not be able to either take up or utilise. These strains, however, are able to grow on *N*-acetylglucosamine as sole source of carbon (Chapter 4, Table 2) and so production of chitinase by *E. americana* is probably not primarily of nutritional significance. Rather, this finding supports the hypothesis that chitinase is significant as a virulence factor in ISN pathogenesis.

SDS-PAGE clearly demonstrated that the 33 kDa chitinase from *E. americana* strain PI98 could be successfully and easily purified from culture filtrates by hydrophobic interaction chromatography. All the activity was extracted from the culture filtrates applied to the phenyl sepharose column utilised, indicating the possibility of high yields, although quantitative analysis of this efficiency was not attempted in this study. Such a method was also found to be highly effective with the cloned *chi*B chitinase from *S. marcescens* strain BJL200, over-expressed periplasmically in both *E. coli* and *S. marcescens* (Brurberg *et al*., 1994). This report found it necessary to utilise isopropanol in the elution buffer to optimise release of the bound enzyme from the hydrophobic matrix. This suggests a strong hydrophobic interaction, which also appears to be the case with the *E. americana* chitinase, where elution was found to occur only in extremely low salt concentrations. Intermolecular or intramolecular hydrophobic interaction may also increase at higher molecular densities. This was manifested by the tendency of enzyme preparations to precipitate

when highly concentrated. Intramolecular hydrophobic interaction may also cause discrepancies in the protein's behaviour in native and SDS-PAGE gels, where dramatic shifts were observed in the enzymes position in SDS-PAGE gels when extensively boiled in the presence of SDS, β-mecaptoethanol and urea. As well as native topology, the exposed hydrophobicity of the enzyme may produce a tendency to bind relatively more SDS, thereby causing more rapid migration in Laemmli SDS-PAGE gels (Anon, 1993).

Affinity chromatography was not effective for the purification of the chitinase from *E. americana*, and did not efficiently extract the enzyme from the culture filtrates tested, even when used in large excess. The major fraction released by alkali treatment of the bound matrix had no chitinase activity, and was poorly soluble. It is possible that this fraction represents a chitin binding protein of possible significance to *E. americana* virulence. Such proteins have been demonstrated to effect the specific attachment to chitin in chitinolytic *Vibrio* species and have been postulated as being important in the ecology of these organisms (Raikhel & Lee, 1993; Montgomery & Kirchman, 1993). Affinity chromatography has been effective in the purification of chitinases from among others, *Aphanocladium album* (Strivastava *et al.*, 1985), *Streptomyces kurssanovii* (Ilyina *et al.,* 1994), *S. marcescens* (Roberts & Cabib, 1982) and *Trichoderma harzianum* (Deane, 1994). In other systems, however, problems related to binding, or specific release of enzyme from chitin may prevent effective purification (Verburg & Huynh, 1991), or result in components of a chitin-binding complex being simultaneously collected (Valadares, 1995).

The chitinase of *E. americana* had an optimum temperature of activity of 50˚C, and was stable at temperatures below 40˚C. Such results are in approximate agreement with those of many other chitinases, and reflect the mesothermic environment occupied by these organisms. Such correlation is also found in

thermophilic microorganisms; hence, the chitinase of Streptomyces *thermoviolaceus* is also found to be highly thermotolerant (Tsujibo *et al*., 1993). The optimum pH for *E. americana* chitinase activity was found to be 6.5, with the enzyme remaining relatively stable between pH 2.0-5.5. These results are similar to that obtained by Deane (1994) for a chitinase from *Trichoderma harzianum*; however, most chitinases are stable between pH 4.0-8.0 (Cabib, 1987). The activity of the enzyme at low pH values (below 4.5) may be underestimated due to the tendency of CM-Chitin to precipitate in high H^+ concentrations (Wirth & Wolf, 1990). High OH^- concentrations, however, do not affect the assay, and the finding that enzyme activity was severely repressed at these pH values may reflect significantly on the catalytic mode of action. This finding may be related to the fact that acid residues are often significant in the active sites of enzymes mediating the acid hydrolysis of glycosidic bonds, including chitinases (Henrissat, 1990; Watanabe *et al*., 1993b). This feature of chitinases could also be the reason for the particular sensitivity of the *E. americana* enzyme to certain divalent cations, which have been shown to bind aspartic and glutamic acid residues in certain enzymes, thereby causing inhibition (see Chapter 6). The particular inhibitory effects of cobalt and manganese ions are probably related to this; and may be exacerbated by specific molecular interactions, related to such factors as ionic radius of the binding metal. Metal ions are probably not required by the *E. americana* chitinase for activity, which is reflected in the resistance of the enzyme to EDTA. The only chitinolytic enzyme to have been demonstrated to have a requirement for a metallic co-factor is that of *Phascolomyces articulosus* (Balasubramanian & Manocha, 1992).

Many further experiments are probably required to fully characterise the chitinase of *E. americana*. Of particular importance to ISN pathogenesis, is the determination of the specificity of the chitinase to various forms of the substrate, both catalytically and in binding terms. In particular, differences in activity towards

crystalline (pre-formed) chitin and nascent chitin should be investigated. The soluble substrates, CM-Chitin or glycol chitin may simulate such nascent chitin, since in these forms, the fibrillar structure is disrupted (Cabib, 1987; Muzzarelli, 1988).

CHAPTER 6
Cloning and sequence analysis of a chitinase gene from *Ewingella americana*

INTRODUCTION

The last decade has seen a burgeoning of interest in the molecular genetics of microbial enzyme production. Chitinases have received much attention, possibly because of their role and potential application in defence against fungal plant pathogens (Schlumbaum *et al.,* 1986; Hedrick *et al*., 1988; Arlorio *et al*., 1992). This has stimulated numerous efforts to clone plant chitinase genes, e.g. in rice, *Oryza sativa* (Zhu & Lamb, 1991) and bean, *Phaseolus vulgaris* (Margis-Pinheiro *et al*., 1991), with the eventual aim of producing plants with increased chitinase activity or the creation of novel, pathogen-resistant transgenic plant varieties. Interest has also focused on the application of bacterial chitinases, which in certain systems have been shown to be directly correlated with biocontrol activity, e.g. *Enterobacter agglomerans* (Chernin et al., 1995). Bacterial chitinase genes have been applied and manipulated for the improvement of biocontrol bacteria and for the development of novel biocontrol systems and models (Shapira *et al.,* 1989; Chet *et al.,* 1993). An excellent example of this is the introduction of a *Serratia marcescens chitinase* gene into *Rhizobium meliloti*, which, during symbiosis on alfalfa roots, was able to confer mycolytic activity against *Rhizoctonia solani* (Sitrit *et al*., 1993). This approach was successful due to the development of a stable p*tac* expression vector for rhizobacteria, which was used to introduce the cloned chitinase gene. Prokaryotic chitinase genes have the potential for such expression in a wide range of both prokaryotic and eukaryotic hosts when fused to appropriate promoter constructs, by

virtue of their lack of introns; but may be challenged by the development of efficient expression systems based on eukaryotic cDNA (Metreaux *et al*., 1989). However, the *S. marcescens chiA* gene has been successfully expressed in tobacco leaves when under the control of photosynthetic gene promoters (Jones *et al*., 1988), and in the same system, further improved by coupling chitinase secretion to plant derived signal sequences (Lund & Dunsmuir, 1992).

Table 1. Chitinolytic enzymes cloned and/or sequenced from bacteria

Gene	Organism	Reference
chiA/chiB	*Serratia marcescens*	(Jones *et al.,* 1986)
chitinase	*Serratia marcescens*	(Fuchs *et al.,* 1986)
chitinase	*Vibrio vulnificus*	(Wortman *et al.,* 1986)
chiA,B,C,D,E	*Streptomyces liquefaciens*	(Joshi *et al.,* 1988)
chitinase	*Streptomyces plicatus*	(Robbins *et al.,* 1988)
chitobiase	*Serratia marcescens*	(Kless *et al.,* 1989)
chitinase	*Saccharopolyspora erythraeus*	(Kamei *et al.,* 1989)*
chiA	*Serratia marcescens*	(Shapira *et al.,* 1989)
chitinase	*Aeromonas hydrophila*	(Roffey & Pemberton, 1990)
chitinase A1	*Bacillus circulans* WL-12	(Watanabe *et al*., 1990)
chitA,B,C,D	*Streptomyces lividans* 66	(Miyashita *et al.,* 1991)
chit63 (chtA)	*Streptomyces plicatus*	(Delic *et al.,* 1992)
chts	*Streptomyces plicatus*	(Robbins *et al*., 1992)
chiD	*Bacillus circulans* WL-12	(Watanabe *et al*., 1992)
exochitinase	*Streptomyces olivaceoviridis*	(Blaak *et al*., 1993)
chitinase	*Alteromonas* sp. O-7	(Tsujibo *et al*., 1993a)
chi40	*Strep. thermoviolaceus*	(Tsujibo *et al.,* 1993b)
chitosanase	*Streptomyces* sp.	(Masson *et al*., 1994)
chitinase	*Aeromonas caviae*	(Sitrit *et al*., 1994)†
chitinase II	*Aeromonas* sp. No. 10S-24	(Ueda *et al.,* 1994)
chi69	*Janthinobacterium lividum*	(Gleave *et al*., 1995)
chitinase	*Kurthia zopfii*	(Ikeda *et al*., 1995)†

* Amino acid sequence; †Unpublished Genbank entry

The majority of bacterial chitinase genes have been cloned by screening plasmid-based genomic libraries in *E. coli*, plated on media incorporating colloidal chitin. Such an approach has proven to be effective in cloning chitinases from *Enterobacteriaceae* such as *S. marcescens* (Fuchs *et al.,* 1986) and other Gram-

negative bacteria such as *Aeromonas hydrophila* (Roffey & Pemberton, 1990) and *Vibrio vulnificus* (Wortman *et al.,* 1986). Many genes derived from Gram-positive bacteria such as *Streptomyces* sp. are poorly expressed in *E. coli,* largely due to promoter incompatibility. Recently, this problem has been largely overcome by the use of expression vectors such as λZAP (Stratagene) which allow expression to be driven by the *E. coli lac* promoter (Robbins *et al*., 1992).

Cloning of a potential virulence determinant provides a powerful tool in confirmation of that character's significance. Such cloned genes give us the opportunity to develop mutants *in vitro* that may then be screened for alterations in pathogenic phenotype (Gulig, 1993). Falkow (1988) has formulated a set of criteria for investigations into the effects of genetic mutation in microbial pathogenicity, known as molecular Koch's postulates, by virtue of their affinity to the original. These assumptions state:

1. A relationship exists between a phenotype/function and pathogenicity.
2. A mutation should be constructed or isolated in the gene encoding the function, forming an isogenic mutant strain, so that the function is eliminated or attenuated.
3. The isogenic mutant strain should be attenuated for virulence in an appropriate model.
4. The mutation should then be complemented by the precisely cloned wild-type allele.

Molecular techniques have previously only been applied to the brown blotch / *P. tolaasii* system, from which genes involved in lipodepsipeptide toxin synthesis (Rainey *et al*., 1993) and phenotypic instability (Grewal *et al*., 1994) have been cloned. Molecular genetics would allow us to confirm the significance of chitinase in ISN pathogenesis, and therefore, an attempt was made to clone this gene from *E.*

americana strain PI98. Additionally, this is the first molecular genetic study involving *E. americana*, which may provide useful information on gene organisation in this organism, as well as to determine the evolutionary affinities of the chitinase at the sequence level.

MATERIALS AND METHODS

Construction of a plasmid / genomic DNA library for E. americana Strain PI98 (=NCPPB 3905)

Genomic DNA (1 µg) prepared as described previously (see Chapter 3) was restriction digested to completion with *Hin*dIII and the products precipitated with propan-2-ol. The phagemid vector, pBluescript II sk+ (Stratagene; 0.5 µg) was similarly digested and precipitated. The DNA precipitates were then each resuspended in 9 µl of distilled water and the products mixed. Ligase buffer was added according to manufacturer's instructions and the DNAs subsequently ligated overnight at 4˚C with T4 DNA ligase (NBL). The ligation mixture was then inactivated by heating at 70˚C for 20 min and allowed to cool to room temperature.

Transformation of E. coli and selection of recombinants

The *Hin*dIII genomic library prepared above was transformed into *E. coli* as described in Chapter 3. Transformed cells were plated on LB agar plates, supplemented with X-gal, IPTG and ampicillin, and incubated overnight.

Screening transformants for expression of chitinase

CM-chitin-RBV (5 mg ml^{-1}) was added to molten 2x LB agar at 50% (v/v). On cooling to 45˚C, 50 µg ml^{-1} ampicillin was added and plates poured. White recombinant colonies from X-gal plates were picked with sterile cocktail sticks and stab inoculated into this medium, with approximately 0.5 cm spacing between applications. Plates were then incubated overnight at 37˚C. Direct plating of primary transformants was found to be possible on this medium and was later used to select chitinase positive subclones.

Preparation of plasmid DNA

Bacterial colonies containing plasmids of interest were picked with cocktail sticks and placed in sterile universal tubes containing 5 ml LB broth with 50 μg ml^{-1} ampicillin. Tubes were incubated at 37˚C overnight with shaking at 250 rpm, after which plasmid DNA was extracted from harvested cell pellets using the "Wizard" plasmid miniprep kit (Promega) according to manufacturer's instructions. Larger scale plasmid purifications were conducted using the Quiagen tip 100 kit (Quiagen).

Subcloning, preparation of deletion mutants and sequencing strategy

A restriction endonuclease map was prepared from a chitinase positive clone, designated pPI13. This was found to carry an insert of 10 kb, which was subsequently reduced to a 3.4 kb *Mbo*I/*Xho*I fragment (pPI23). Subclones were prepared utilising available restriction sites, and the products screened for production of chitinase as before. Care was taken to ensure that regions overlapping the main restriction sites could be sequenced. DNA fragments were cloned into pBluescript II ks+ (Stratagene), so that the complementary DNA strand could be prepared for sequencing. Regions lacking suitable restriction sites were subjected to unidirectional deletion with exonuclease III.

Construction of unidirectional nested DNA deletions with exonuclease III and mung bean nuclease

Unidirectionally deleted subclones were constructed according to a modification of the method of Henikoff (1984). Purified vector DNA (5 μg) containing suitable restriction sites was doubly digested with enzymes *Sac*I (Exo III protective) and *Xba*I (Exo III permissive) in a final volume of 50 μl. For 4 time points, 4 tubes were set up, each containing 37.5 μl 1.35 x mung bean nuclease buffer made from a x 10 stock (300 mM sodium acetate (pH 4.5), 500 mM NaCl, 10 mM $ZnCl_2$, 50% glycerol (v/v)). Exonuclease III (300 U; Promega UK) was added to the digested DNA without buffer adjustment at 37˚C, and the first aliquot of 12.5 μl removed after 1.5

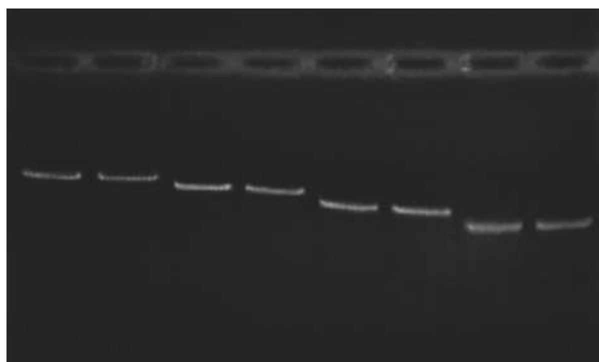


Figure 1. Ethidium bromide-stained agarose gel of the products of exonuclease III digestion of previously restricted plasmid. Each deletion step was approximately 250 bp under the conditions used (see text; molecular weight markers not shown).

min and mixed with mung bean buffer in a labelled tube. Subsequent aliquots were removed from the Exo III digest at 1 min intervals. Mung bean nuclease (5 U) was then added to each tube and the mixture incubated for 30 min at 30˚C. The material from each time point was purified by agarose gel electrophoresis (Figure 1) and the DNA recovered by the Geneclean process (Bio 101 Inc.) according to manufacturer's instructions. Products were then ligated, transformed into *E. coli* XL1-Blue and single stranded DNA prepared as described previously.

DNA Sequencing

Single stranded DNA was subjected to dideoxynucleotide, chain termination sequencing reactions (Sanger *et al*., 1977) using the Sequenase II kit (Amersham) and [α-^{35}S]dATP as label. Two sets of reactions were set up for each template, according to manufacturer's protocols; one incorporating dGTP into the growing DNA chains and another, incorporating dITP in order to eliminate electrophoretic compression problems. Dimethyl sulphoxide (1 µl) was also included in the dITP labelling reactions to relax DNA secondary structure, thereby reducing polymerase associated pausing artefacts. Electrophoresis of the reaction mixtures was as described in Chapter 4.

Assembly of chitinase sequence data

Sequencing gels were read on two separate occasions in order to reduce transcriptional errors. Sequence data were assembled using GeneJocky software

(Biosoft) running on an Apple Macintosh microcomputer. Data from overlapping subclones, and both DNA strands was compared and anomalies rationalised either by re-reading gels, or repeating sequencing reactions.

Analysis of sequence data

E. americana sequence data was compared to previously published chitinase sequences, initially using GeneJocky software. On-line searches of the EMBL Nucleotide Sequence Datalibrary were conducted using the FASTA algorithm (Pearson & Lipman, 1988). Similar on-line searches of the SWISSPROT database were conducted with deduced peptide sequences using the BLAST algorithm (Altschul *et al*., 1990) with HSPcrunch filtering (Sonnhammer & Durbin, 1994), and on deduced amino acid composition with PROPSEARCH (Hobohm *et al*., 1994). More detailed alignment / homology analysis was carried out with the Wisconsin Genetics Computer Group GCG suite of programs, the Staden suite and ClustalW (Thompson *et al*., 1994). All programs were used with default settings.

Determination of chitinase gene copy number

Southern blots were set up as described in Chapter 4 and filters probed with a Biotin-14-dATP labelled fragment comprising the *Bam*H1 / *Xho*1 region of the chitinase positive construct pPI23.

RESULTS

Library screening and selection of transformants

In a screen of 200 *E. coli* DH5α transformants harbouring recombinant pBluescript (selected by the appearance of a white colonial phenotype on X-Gal/IPTG plates), 1 colony was found to produce a 12 mm clearing zone after overnight incubation on the chitinase screening medium. The chitinase positive clone, designated pPI13 was then subjected to deletion analysis. A 3.2 kb *Xba*1 / *Xho*1 subclone of pPI13 (designated pPI23) was constructed, producing qualitatively greater clearing zones

on the screening medium than the parent construct; this was then used in subsequent studies.

Copy number of the cloned chitinase gene in the E. americana genome.

Southern hybridisation, using the *Eco*0109I / *Xho*I fragment of pPI23 (Figure 4; representing the complete chitinase open reading frame) as a probe, suggested that the cloned chitinase gene was probably present in single copy in the strains tested (Figure 2), including the type strain of *E. americana,* NCTC 12157.

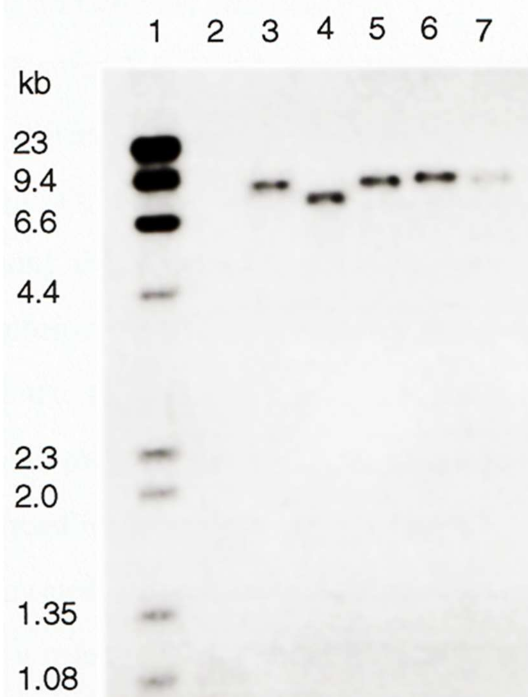


Figure 2. Southern hybridisation analysis of *Eco* R1 restricted genomic DNA probed with a biotin-14-dATP labelled *Eco*0109I / *Xho*I fragment of pPI23. Filter was washed twice for 15 min at 68˚C, 0.1x SSC. The detection control was a pre-biotinylated mixture of *Hin*dIII digested λ DNA and *Hae* III digested ϕX174 DNA (lane 1) and negative hybridisation control, 1 µg of a 1kb Ladder (Gibco-BRL). Lanes 3, 4, 5 & 6 contained genomic DNA from ISN strains PI74, PI98, PI145, PI180 respectively. Lane 7 contained genomic DNA from *E. americana* NCTC 12157.

With the exception of strain PI98, the probe hybridised to a *Eco*R1 genomic fragment corresponding to approximately 9.0 kb. In strain PI98, however, the chitinase probe hybridised to a band of approximately 8.0 kb, representing minor polymorphism within this locus in this strain. The above evidence reinforces the

close links of ISN strains with the type-strain of *E. americana*, a clinical isolate, as demonstrated in Chapter 4 using the 16S rDNA probe. No other bands were visible, even on extended exposure to X-ray film, indicating the probable absence of close homologues to the probe in the *E. americana* genome. The possibility cannot be excluded, however, that lower hybridisation stringencies might detect other homologues.

Restriction mapping and deletion analysis

Deletion analysis (Figures 3 & 4) indicated that the minimum span of insert DNA required for efficient chitinase production in *E. coli* is the *Eco*0109I / *Xho*I fragment of pPI23, incorporated in pPI31, which produced the largest clearing zones among the various deletants. This phenomenon is possibly related to copy number of the truncated plasmid. In contrast to pPI1, pPI31 is oriented in opposition to the vector *lacZ* promoter, indicating that transcription of chitinase in *E. coli* probably occurs via *E. americana* promoter elements. Plasmid pPI21, formed by deleting the region from the *Eco*RVsite of pPI23, produces highly truncated, but detectable clearing zones on chitinase screening plates. In contrast, a deletion from the *Eco*RV site to the *Xba*I site of pPI23 produces a construct devoid of chitinase activity. Similarly, pPI30, containing the 329 bp *Eco*0109I / *Eco*RV fragment of pPI23 is also inactive. This result is somewhat surprising in the light of the activities of pPI21 and pPI31.

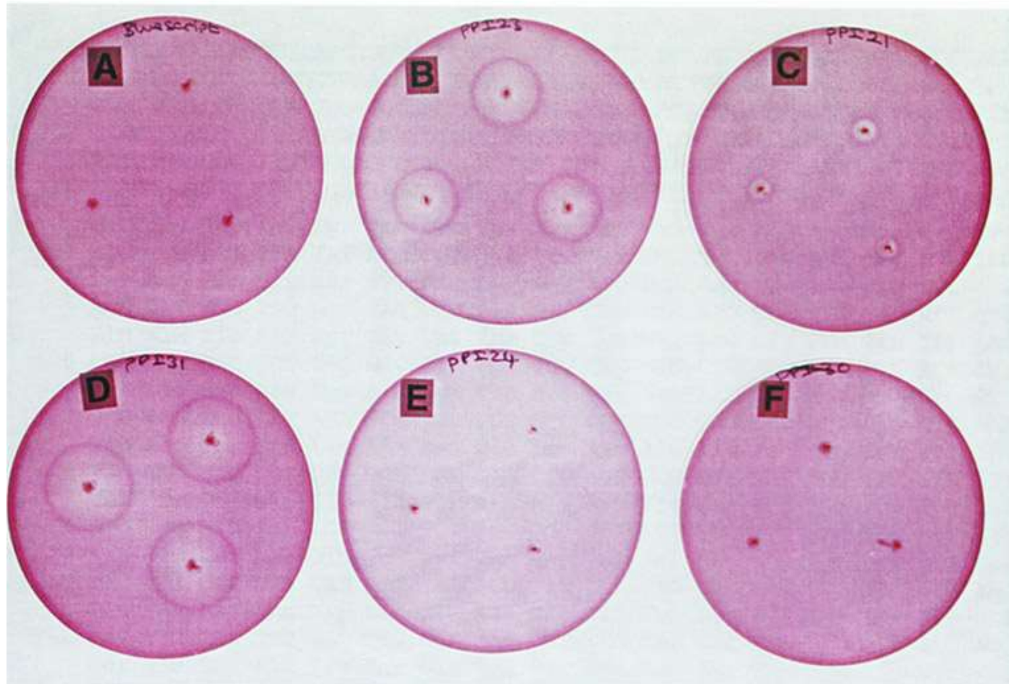


Figure 3. Clearing zones produced by *E. coli* DH5α harbouring various plasmid constructs, after overnight incubation at 37˚C on LB agar supplemented with ampicillin and CM-chitin-RBV. A. Control - pBluescript, no insert; B. pPI23; C. pPI21; D. pPI31; E. pPI24; F. pPI30.

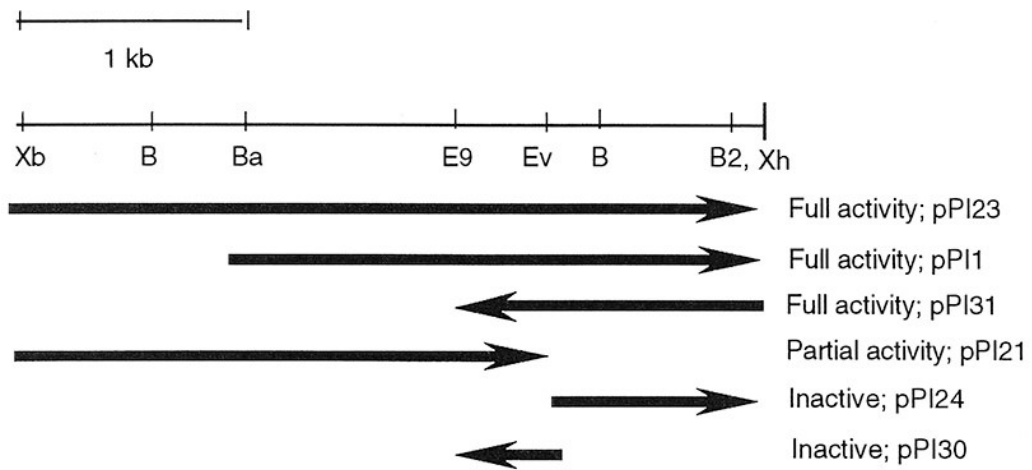


Figure 4. Partial restriction / deletion map of insert regions of pPI23 and subsequent sub-clones. Vector DNA is pBluescript II sk+ (2.96 kb). Restriction sites: B, *Bgl*I; Ba, *Bam*HI; B2, *Bgl*II; E9, *Eco*0109I; Ev, *Eco*RV; Xb, *Xba*I; *Xho*I, Xh. Activity estimated from zone sizes produced on LB + RBV CM-chitin + ampicillin agar. Arrows indicate the direction of the pBluescript *lac*Z promoter.

```
   1 CTGGGCTACGAGGGGCACGTGGTCTCTTCCGCGCTCTGTTCGCCTTCCACGAAATGACCCTGCCAGA
  68 TAGCTACGGCGACCCGGTAAACCAGTTCTGCGATCAGCCCGATTTTCACCGCCGCGCGACCGCTTTG
 135 CGCTTTCACGCCCTAATGGCGGCTATGATCTGGTGGCGGCGATGATTGAGCAGGGCGCGCACCACGT
 202 CTTCGCCCCGCGTTCACCAGTAATGGGATAAACATTTAAAACTATCTAACTGTTTTATATCGTTTTA
 269 ATTTTGATGCTGTTGTGCCAGCGGTGCTACTACCCTGATCGAATGCATCAAAAAAGGTTGTAGGAGA
 336 AGATGACTTTGCCGCCGGAGAAGGCCCCGGCGTAAACGTTCTTACCTACATAAAAAGGATATAAAAC
     Met Ala Thr Leu Tyr Thr Pro Tyr Ile Asp Val Ser Leu Asn Ala Leu Trp  17
 403 ATG GCT ACT TTA TAT ACC CCT TAC ATT GAT GTG TCC CTG AAT GCG CTG TGG
     Ser Asp Trp Gln Asn Tyr Pro Lys Gly Arg Pro Asn Pro Ile Tyr Ser Gln  34
 455 AGT GAC TGG CAG AAC TAC CCT AAA GGT CGC CCA AAC CCA ATT TAC TCT CAA
     Gln Ala Ile Ser Tyr Gly Val Asp Gly Leu Val Leu Gly Phe Leu Thr Leu  51
 506 CAG GCG ATT TCT TAC GGC GTT GAT GGT CTG GTG CTG GGC TTC CTG ACA CTT
     Ser Pro Ser Asn Lys Ala Cys Trp Ala Ala Ser Asp Ala Met Pro Leu Glu  68
 557 TCC CCG TCG AAT AAA GCC TGC TGG GCC GCC TCC GAC GCC ATG CCG CTG GAA
     Trp Ala Leu Pro Leu Ala Asn Glu Leu Asn Ala Ala Asn Arg Gln Val Ile  85
 608 TGG GCG CTG CCG CTG GCA AAC GAG CTG AAC GCG GCA AAC CGT CAG GTA ATT
     Val Ser Phe Gly Gly Ala Ser Asn Ala Asp Ile Ser Thr Lys Phe Thr Val  102
 659 GTC TCC TTC GGC GGC GCG TCT AAC GCC GAT ATC TCC ACC AAG TTC ACC GTT
     Asp Gln Leu Val Gln Thr Tyr Thr Asp Val Val Gln Lys Phe Lys Ala Lys  119
 710 GAC CAG CTG GTA CAG ACT TAC ACC GAC GTT GTG CAG AAG TTT AAA GCC AAG
     Gln Leu Asp Phe Asp Leu Glu Asn Gly Gln Tyr Asp Tyr Asn Lys Ile Ser  136
 761 CAG TTG GAC TTC GAC TTG GAA AAC GGT CAG TAC GAC TAC AAC AAA ATC AGC
     Ser Ala Leu Ala Ile Phe Gln Lys Ser Asn Pro Ser Val Arg Ile Ser Phe  153
 812 TCA GCC CTG GCG ATT TTC CAG AAG AGC AAC CCA TCG GTT CGC ATC AGC TTT
     Thr Leu Pro Thr Met Pro Ala Gly Leu Val Thr Glu Gly Leu Asn Ile Ile  170
 863 ACG CTG CCA ACC ATG CCA GCA GGT TTG GTC ACT GAA GGT CTG AAC ATC ATT
     Gln Ser Ala Lys Asn Ala Gly Val Lys Phe Ser Val Asn Gly Met Ala Met  187
 914 CAG AGC GCC AAA AAT GCC GGT GTG AAG TTC AGC GTA AAC GGT ATG GCG ATG
     Asp Tyr Asn Asp Gly Val Ser Ser Gln Asp Met Gly Lys Ala Ala Thr Ser  204
 965 GAC TAT AAC GAC GGC GTT TCC AGC CAA GAT ATG GGT AAA GCA GCG ACC AGC
     Ala Ala Thr Ser Ile Lys Thr Gln Leu Gly Lys Leu Tyr Pro Gly Gln Thr  221
1016 GCG GCA ACC AGC ATC AAA ACC CAG CTG GGC AAA CTT TAT CCG GGC CAG ACT
     Asp Ala Gln Leu Tyr Pro Leu Val Ala Ile Thr Pro Met Ile Gly Leu Asn  238
1067 GAC GCA CAG CTT TAC CCA CTG GTA GCC ATC ACC CCG ATG ATC GGT CTG AAC
     Asp Asp Thr Thr Glu Met Phe Lys Leu Pro Asp Ala Thr Thr Val Ala Thr  255
1118 GAC GAC ACC ACC GAG ATG TTC AAA CTG CCA GAC GCC ACC ACC GTT GCC ACT
     Phe Ala Lys Gln Lys Asn Met Ala Phe Ile Gly Ser Trp Ser Phe Asn Arg  272
1169 TTC GCC AAG CAG AAA AAC ATG GCG TTT ATC GGT AGC TGG TCG TTT AAC CGC
     Asp Asn Pro Ser Ser Tyr Thr Tyr Val Asp Leu Gln Thr Ser Ser Asn Pro  289
1220 GAC AAC CCA TCG AGC TAC ACC TAC GTT GAT TTG CAG ACC AGC AGT AAC CCG
     Ala Gln Lys Ala Ser Gly Asp Tyr Ala Lys Thr Phe Leu Ala Gly Ile Arg  306
1271 GCG CAG AAA GCC TCC GGT GAT TAT GCG AAG ACG TTT TTA GCG GGT ATT CGT
     Stop                                                                 306
1322 TAA GGGGAAATAGTGGGCAGGGAGGCCCACAGCTCCTGTTTAGATCTTTAAATTCAAAAACCGTGG
                                                                    >>>>

1388 GCTCGCCGCCCACACTTAGGTTTTTTAAATTCAAAACCGTGGGCTCGCCGCCCACACTTAGGTCTTT
     >>      <<<<<<                        >>>>>>      <<<<<<

1455 TAAATTCAAAACCGTGGGCTCGCCGCCCACACTTAGGTCTTTAAATTCAAAACCGTGGGCTCGCCGC
                     >>>>>>      <<<<<<                      >>>>>>     <<

1522 CCACACTGGCCCAAAGGTTTTAAACGAAACCCTTTGGAATCCTGCGTTTTTTTGTTGTTGTAAGATC
     <<<<

1589 GAGGCTTCGCTGGGTTGGGATGGACATGTTTCAGCAGCTTCTATG
```

Figure 5. Full nucleotide sequence and peptide translation of the *chi*A gene from *E. americana* strain PI98. The putative Shine-Dalgano sequence preceding the ATG start codon is double underlined. Four inverted repeat regions (<>) within four direct repeats (Dotted underlining; anomalous **A** in bold), constituting a possible novel terminal region (See text). The nucleotide sequence has been deposited in the EMBL/Genbank datalibraries under accession number X90562.

Nucleotide sequence analysis

DNA sequence analysis of the assembled region from the *Bam*H1 to the *Xho*1 sites of pPI1, revealed an open reading frame (ORF) of 918 bp, with an ATG translation initiation codon at 403 bp and a TAA stop codon at 1322 bp (Figure 5). This *E. americana* chitinase gene was subsequently designated *chi*A. A putative Shine-Dalgano sequence (AGGA) is located 9 nucleotides 5' of the ATG codon, which is consistent with the general range found in prokaryotes (7-11; Gold *et al*., 1981). The ORF has a nucleotide base composition of 53.5% G+C, which agrees closely with the reported genomic base composition of *E. americana* (54.4%; Grimont *et al*., 1983). Additionally, there is a bias of 62.7% G or C for the third nucleotide in each codon. The general organisation of this gene is represented in Figure 7. Analysis of sequences upstream of the SD site with the Staden program NIP, did predict some likelihood of *E. coli*-type promoter elements occurring from approximately base 200 (Figure 6). However, motifs exactly matching the *E. coli* σ^{70} recognised consensus could not be found.

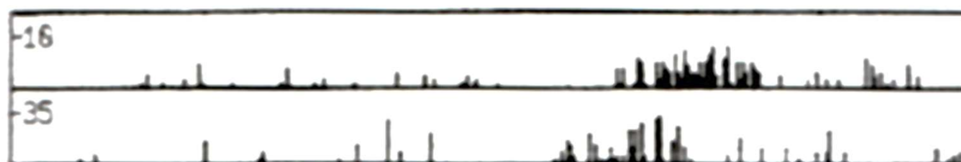


Figure 6. Output from the Staden program, NIP, predicting the occurrence of possible -10 and -35 promoter elements, within the *E. americana chi*A gene, from 1 to 402bp.

Searches of the general and prokaryotic databases of EMBL and Genbank revealed a significant match with the nucleotide sequence of Chitinase II from *Aeromonas* sp. (accession number D31818; Ascii), with an optimised FASTA score of 405. The next best match was with an unspecified *E. coli* chromosomal region (U18997), having an optimised FASTA score of 116. There were no further

matches with any other chitinase genes in the current versions of the above databases, at the DNA level.

Sequences downstream of the TAA stop codon demonstrate a highly unusual structure of four direct repeats of the sequence:

TTTAAATTCAAAACCGTGGGCTCGCCGCCCACACTTAGG

>>>>>> <<<<<<

Within this repetitive motif is a palindromic element, potentially forming a stem-loop structure. However, there is no poly-thymidine element immediately following such structures, which would be typical of bacterial *Rho* independent transcriptional terminators. The first direct repeat contains an additional adenosine, when compared to subsequent repeats, which is marked in bold in Figure 5. The spacer bases between repeats were also found to vary slightly; so that the first spacer reads TT, the second, CT and the third, C. Sequences downstream from the TAA stop, and translated in all three reading frames, show no further similarity to any published chitinase sequence.

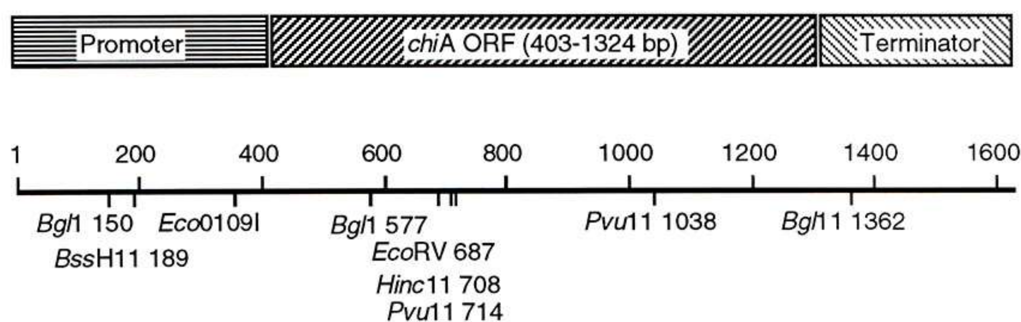


Figure 7. Organisation of the *E. americana chiA* chitinase gene.

Analysis of the deduced amino acid sequence of chiA

The *E. americana* strain PI98 chitinase open reading frame would code for a protein of 306 amino acids with a molecular weight of 33200 Da, with composition:

Amino acid		Count
Ala	:	34
Arg	:	5
Asp	:	20
Asn	:	20
Cys	:	1
Glu	:	5
Gln	:	18
Gly	:	19
His	:	0
Ile	:	15
Leu	:	26
Lys	:	18
Met	:	9
Phe	:	13
Pro	:	16
Ser	:	28
Thr	:	23
Trp	:	5
Tyr	:	14
Val	:	17
Other	:	0

Molecular weight: 33200.43 Da

Analysis with the GCG program "Signal scan" of this peptide sequence (translated in Figure 5) did not predict the presence of a typical N-terminal signal peptide, as indicated by the presence of a positively charged region, a hydrophobic region and a signal-sequence cleavage site (von Heijne, 1983). The absence of such a structure at the N-terminus is also demonstrated by the hydropathy scales presented in Figure 8.

Searches conducted against the SWISSPROT peptide database using the BLAST program revealed that the *E. americana* chitinase was most homologous to the chitinase (EC 3.2.1.14) from *Saccharopolyspora erythraeus* with a BLAST score of 166 (Perfect match = 2328); 7.8% identity. FASTA searches did not return a chitinase as a significant match in the 50 most homologous sequences. Although not presently in the SWISSPROT database, the chitinase II from *Aeromonas* sp. (Ueda *et al*., 1994) displayed greatest homology with the *E. americana* sequence,

when the full peptide sequences were aligned with ClustalW (33% amino acid match; Figure 8). Significantly, searches using the PROPSEARCH program (Hobohm *et al*., 1994) with the deduced amino acid composition of ChiA revealed matches with several chitinases and chitinase-related proteins such as endo-β-N-acetylglucosaminidase, endo-1,4-β-xylanase, chitosanase and glucan endo-1,3-β-glucosidase.

The specially conserved amino acid motifs, present in chitinases and chitinase-like proteins, and believed to be involved in catalysis (Henrissat, 1990; Gilkes *et al*., 1991; Watanabe *et al*., 1993b) were also present in the *E. americana* sequence (Boxed in figure 9; region 1 & region 2). Watanabe *et al*. (1993b) classified prokaryotic chitinases on the basis of sequence homology within the complete catalytic domain. Group A chitinases included those of *B. circulans* (Chi A1), *S. marcescens* (ChiA), *Str. lividans* (Chi C), *Str. plicatus* (Chitinase 63) *and Alteromonas* sp. (Chitinase 85). Group B chitinases included *B. circulans* (Chitinase D), *Str. lividans* (Chitinase A) and *Str. lividans* (Chitinase B). Group C chitinases included only that of *Str.* (now *Saccharopolyspora*) *erythraeus* . Later work by Ueda *et al*. (1994) placed the chitinase II of *Aeromonas* sp. No. 10S-24 within group C on the basis of divergence from chitinases of the other groups and similarity of domain organisation (see later). The chitinase of *E. americana*; on the basis of homology to Chitinase II from *Aeromonas* sp., is therefore classified as a group C prokaryotic chitinase. This system contrasts with the plant chitinases which display strong sequence homology, and may also be the case with the filamentous fungal chitinases sequenced so far (Valadares *et al*., submitted for publication).

Figure 8. Alignment of *E. americana* ChiA protein sequence with chitinase II from *Aeromonas* sp. No. 10S-24, generated by ClustalW, with hydropathy scales calculated by SeqVu 1.0 (Garvan Institute of Medical Research). Identical residues are boxed, and regions of hydrophilic and hydrophobic amino acids indicated by blue and red scales respectively. The signal sequence cleavage site in the *Aeromonas* sequence resides between sites 31 and 32.

```
E. americana    1   -----------------MATLYTPYIDVSLNALWSDWQNYPKGRPNPIYSQQAISYGVDGLVLGFLTLSPSNKAC  58
Aeromonas II    1   MSRKSQSRNLRRLGMGALALAMGLASVGSLATEAAPYFFTWGYGESSYKFTSLVDAKQKAGLDAATLAFIITGS  74

E. americana   59   WAASDAMPLEWALPLANELNAANRQVIVSFGGASNADISTKFTVDQLVQTYTDVVQKFKAKQLDFDLENGQYDY  132
Aeromonas II   75   SGCSLSNDVDNMLSDVLAFQQQGGRLIISFGGAAVPMWKPAVPAPRWPRWWMPCCNAPACVPLDFDIEGSQLSQ  148

E. americana  133   NKISSAL--AI--FQKSNPSVRISFTLPTMPAGLVTEGLNIIQSAKNAGVKFS-VNGMAMDYNDGVSSQD-MGK  200
Aeromonas II  149   TALNSVRNEAIKTLQAKYPDLYISFTLPTNPTGLTSEGLNLLRGAQAAGVRTDVVNVMTMDYGASISSGKKMGD  222

E. americana  201   AATSAATSIKTQLGKLYPGQTDAQLYPLVAITPMIGLNDDTTEMFKLPDATTVATFAKQKNMAFIGSWSFNRDN  274
Aeromonas II  223   LAVQAGNSLFTQLKGIYTTKSDAELWAMVGITPMIGKNDVQTEIFTTADATQVANFAKQYNIGLLSFWALQRDR  296

E. americana  275   PSSYTYVDLQTSSNPAQKASGDYAKTFLAGIR                                           306
Aeromonas II  297   SGSGSLDDYSLTPQTDYQF-YKAAKTGTPSATPVPTATPAPTATPKPTATPAPTATPVPTGTPTVTPKPTTTPV  369

Aeromonas II  370   PTTAPTATPKPTATPVPSSTTCAATWSSSTAYNGGATVAYNGHNYQAKWWTQGNVPSSSTGDGQPWADLGVCGG  443

Aeromonas II  444   DPVPTATPAPTATPLPTTAPSATPKPTATPVPTSVPTATPAPTVTPVPGGCAAWAEGNTYTAGTCASYGGKDYV  517

Aeromonas II  518   AQVTHTAYVGANWNPAATPTLWKLK                                                  542
```

Figure 9. Multiple alignment generated by ClustalW, of the conserved catalytic region of chitinase and chitinase like proteins. Identical residues are boxed, and cumulative hydrophilic and hydrophobic regions indicated by blue and red scaled shading respectively (generated by SeqVu 1.0 - The Garvan Institute of Medical Research). Numbering refers to amino acid positions in complete proteins.

Most bacterial chitinases sequenced so far appear to be composed of several distinct domains (Watanabe *et al*., 1993a). These include the catalytic domain discussed above, at least one chitin binding domain and one or more copies of the Type III homology unit of fibronectin (Watanabe *et al*., 1994). On this basis, only the catalytic domain could be identified in the *E. americana* sequence, which, with the exception of the Chitinase II from *Aeromonas* sp. and *S. erythraeus* chitinase, displays low homology to the other catalytic domains of other chitinolytic enzymes.

DISCUSSION

This study is the first to report the cloning and heterologous expression of a gene from *E. americana*. Evidence that this gene encodes a chitinase is provided by the expressed proteins ability to readily hydrolyse CM-Chitin-RBV. Additional evidence is provided by recognition of a conserved motif within the deduced amino acid sequence of this gene, which is known to be associated with chitin degradation. The deduced size of the protein translated from *chi*A is in agreement with the size of the chitinase whose purification and characterisation were reported in Chapter 5 (33 kDa).

The lack of a specialised chitin binding domain in the *E. americana* chitinase probably accounts for the failure of affinity chromatography as a protein purification method in this case. Such a domain has been implicated in the affinity of the enzyme for the insoluble forms of chitin. Deletion of this domain in the chitinase A1 of *Bacillus circulans* resulted in reduction in activity by more than one half in colloidal chitin hydrolysis. Deletion of the Type III fibronectin homology units from this enzyme did not affect colloidal chitin binding, although did reduce activity, again,

by about half. Such deletions did not have an effect on the hydrolysis of soluble carboxymethylated chitin, indicating that the function of the Type III units is to assist in efficient hydrolysis of sufficiently acetylated and therefore insoluble chitin once the enzyme becomes bound via the binding domain (Watanabe *et al*., 1993a). We may speculate on the evolutionary implications of the domain structure of the *E. americana* chitinase on its potential role as a virulence factor in ISN pathogenesis. The work of Mol & Wessels (1990) demonstrated that the chitin in elongating mushroom stipe hyphae is not well crystallised, leading to its particular susceptibility to enzymatic degradation. The vulnerability of nascent chitin was earlier noted by Molano *et al*. (1979), who studied the activity of wheat germ chitinase. This effect is probably related to the accessibility of the chitin chains to attack prior to the point where the chains strongly aggregate to form microfibrils (Cabib, 1987). It is probable that the lack of a special chitin-binding domain in the *E. americana* chitinase is not significant for the efficient hydrolysis of such nascent chitin. Furthermore, Type III fibronectin homology units are also unlikely to be necessary to assist such catalysis. The chitinases of *S. marcescens* (A&B), *Streptomyces thermoviolaceus* and *Janthinobacteriun lividum* also lack Type III units, although do possess binding domains, and so it would be interesting to analyse the relationship between such domain structures and their possible biological function. In this respect, the ChiA chitinase of *S. marcescens* has been shown to display effective antifungal properties (Ordentlich *et al.*, 1988), but has not, so far, been functionally dissected in order to determine domain significance. Plant defence chitinases may also be good examples, where particular antagonistic effects against fungal pathogens are noticed at their hyphal tips (Arlorio *et al*., 1992). As vegetative hyphae mature, then so does their resistance to chitinase degradation; in part, due to the chitin becoming embedded in a matrix of β-glucans and secondarily, by the

chitin aggregating to form fibrillar structures and developing cross-links with the β-glucans.

Alignment of the deduced peptide sequence with other chitinases revealed the unusual nature of the *E. americana* enzyme. The sequence is most similar to that of Chitinase II from *Aeromonas* sp. No. 10S-24 (Ueda *et al*., 1994) and to a lesser extent, the chitinase from *Saccharopolyspora erythraeus* (Kamei *et al*., 1989). As such, the enzyme is classified in group C of prokaryotic chitinases (Watanabe *et al*., 1993b), but displays significant differences. Low levels of homology were observed between the *E. americana* sequence and that of *chi*A and *chi*B from *Serratia marcescens*, indicating that such chitinase homologies might not correlate with taxonomic affiliation of the producing microorganism, although *Aeromonas* is a member of the *Vibrionaceae*, which is closely related to the enteric group. *S. erythraeus* on the other hand is taxonomically distant from *E. americana*. The domain organisation of chitinases presents an interesting evolutionary problem, since each functional unit appears to demonstrate different levels of homology with other comparable domains independently of the enzymes overall taxonomic affinities. Thus, the catalytic domain of the *E. americana* chitinase is most similar to that of Chitinase II of *Aeromonas* sp., but completely lacks a comparable chitin binding domain and Type III units common in several other bacterial chitinases. This situation also occurs in other enzyme families involved in structural polymer degradation, such as the cellulases. Sequencing of the *cel*V gene from *Erwinia carotovora* (Cooper & Salmond, 1993) revealed a typical signal sequence, a catalytic domain which was homologous to that of family A cellulases, such as *cel*Z of *E. chrysanthemi* and several *Bacillus* spp. cellulases; and a cellulose binding domain, homologous to that of several diverse families of cellulases. This, and the data from the chitinases, provides support for the "mix and match" hypothesis for enzyme domain evolution, which may have particular relevance in bacterial

enzymes due to these organisms' tendency for promiscuous genetic exchange. Sequencing of further, more diverse prokaryotic chitinases should assist in clarifying the ancestral origins of these enzymes.

The promoter region of the *E. americana chi*A gene demonstrates A+T richness, typical of many prokaryotic promoters, although definite and linked -10 and -35 signals could not be identified. It is possible that this promoter is of the type recognised by alternative sigma factors, such as the stationary-phase σ^S class (Tanaka *et al*., 1993). Such promoters have been shown to be particularly common in genes associated with expression of virulence determinants (reviewed by Loewen & Hengge-Aronis, 1994). Direct repeat elements have been found overlapping the RNA polymerase binding regions of chitinase promoters of several *Streptomyces* sp. (Delic *et al.,* 1992; Fujii & Miyashita, 1993) and are thought to be important in carbon catabolite control of these genes at the transcriptional level. No direct repeating elements could be detected within the *chi*A promoter region, which is consistent with the constitutive expression of chitinase in *E. americana*. Detailed promoter mapping and timed expression studies are therefore required in order to elucidate the exact site of initiation of transcription, and to determine the precise molecular-genetic basis of chitinase regulation in this case.

Evidence from the subcloning and deletion studies suggests that the *E. americana* promoter is recognised by *E. coli*, since chitinase is produced independently of the orientation of the gene in relation to the vector *lac*Z promoter and in the absence of inducing IPTG. Construct pPI31 produced greater chitin clearing zones than pPI1 and a variant of the latter, designated pPI2, produced by force cloning the insert into pBluescript II ks+ and therefore in reverse orientation (data not shown). This greater activity may be due to increased plasmid copy number, but may also be due to deletion of regulatory regions of the *chi*A promoter. The *Eco*0109I site used in construction of pPI31 resides at base 335 of the full

sequence and the ATG start codon of the chitinase open reading frame at position 379. The sequence preceding this start codon thus reads: CCCCGGCGTAAACGTTCTTACCTACATAAAAAGGAT ATAAAAC**ATG.** Such a region would seem minimal for a putative promoter region and is not the most likely position for -35 and -10 signals as predicted by the Staden program NIP, for the sequence upstream of the ATG start. The possibility cannot be excluded that fusion with vector sequences might produce an efficient hybrid promoter in this case. Similarly, it was reported that expression of a *Aeromonas hydrophila* chitinase ORF in *E. coli* JM109 could be promoted by leaky use of the T7 promoter by *E. coli* RNA polymerase (Roffey & Pemberton, 1990). The observation that pPI21 produces partial chitinase activity and pPI30 is inactive is somewhat paradoxical, since both constructs share identical sequence downstream of the *Eco*0109I site. Additionally, pPI31 lacks sequence upstream of this site and is highly active. A possible explanation for the failure of pPI30 to display activity could be related to the effect of DNA topology in transcription (reviewed by Dorman, 1994; Dorman, 1995) in this minimal size of insert. DNA topology may be altered in pPI21, thereby allowing transcription of the truncated chitinase gene, possibly via the action of an alternative σ factor to that involved in transcription of the mutant (deletion) promoters of pPI30 and pPI31.

The activity of pPI21 is interesting when the amino acid sequence of the truncated polypeptide is examined. The deletion at the *Eco*RV site means that the enzyme lacks sequence from amino acid 95 onwards and removes the entirety of region 2 in Figure 9. The aspartic and glutamic acids of region 2 have been implicated as forming the putative active site of chitinases (Henrissat, 1990; Kuranda & Robbins, 1991; Watanabe *et al*., 1993b). This was suggested as being the case since the above amino acids can promote the acid catalysis of glycosidic bonds and are highly conserved in these positions. Previous evidence involving

chemical modification (Lin & Koshland, 1969) and site-directed mutagenesis (Malcolm *et al*., 1989) of the acid residues of hen egg white lysozyme indicated the importance of aspartic and glutamic acid residues in both chitinase and muramidase activities. Watanabe *et al.* (1993a, b) substituted the conserved serine in region 1 for a threonine residue in *Bacillus circulans* chitinase A1, and observed that 65% of the activity against carboxymethylated chitin was retained. Substitutions to cysteine and alanine caused retention of activity of between 92 and 98%. Conversely, mutations in one of the aspartic acid residues of region 2 to asparagine caused activity to decrease by 75%. The importance of region 2 has also been emphasised by recent crystallographic studies of *S. marcescens* ChiA, when in complex with chitotetraose (Perrakis *et al*., 1994) and of the bifunctional chitinase / lysozyme from *Hevea* sp., hevamine, when in complex with an inhibitor (Vanscheltinga *et al*., 1994). The implications for deletion of all of region 2 of the *E. americana* enzyme mean that additional sites on the truncated enzyme probably have catalytic potential. Several aspartic and glutamic acids precede region 1 in the ChiA protein, but none are as clustered as those of region 2. Further work here is necessary to determine whether these secondary carboxylic groups are important and whether these alter the substrate specificity of the modified enzyme. Evidence for the importance of acid residues in the action of *E. americana* chitinase is provided by data in Chapter 5, where divalent metal ions were found to strongly inhibit activity. Such metal ions are known to form stable complexes with carboxylic groups at the active sites of enzymes (Perkins *et al*., 1979; cited in Milewski *et al*., 1992).

The lack of a typical N-terminal signal peptide in the *E. americana* chitinase is similar to the situation in chitinase B of *S. marcescens*-BLJ200 (Brurberg *et al*., 1995), which is secreted into the periplasm *in vivo*, but remains in the cytoplasm when expressed in *E. coli*. Here, in addition to the lack of a typical signal peptide, structures homologous to other proteins whose export is not accompanied by post-

translational processing, such as the *E. coli* α-haemolysin, could not be identified. A metalloprotease (Nakahama *et al*., 1986) and an extracellular lipase (Akatsuka *et al*., 1994) from *S. marcescens* have also been shown to lack N-terminal signal peptides. Such a situation, however, is extremely unusual and the vast majority of proteins that are efficiently translocated to the medium have signal sequences that are subsequently cleaved during secretion (Pugsley & Schwartz, 1985; Pugsley, 1993). It is possible that part of the *E. americana* chitinase sequence is targeted to the plasma membrane and subsequently released by gradual leakage. Alternatively, the protein may function most efficiently at the host / bacterium interface, again assisted by anchorage into the outer membrane. Whatever the case, the early production of clearing zones on screening plates and in liquid cultures, implies that putative secretory signals or mechanisms are possibly recognised by *E. coli*, as well as *E. americana*. However, this needs to be confirmed by detailed cell fractionation experiments. To assist in the clarification of some of these points, the exported form of chitinase should be subjected to N-terminal sequencing, in order to confirm the absence of processing in this region. It is interesting to note that both the N- and C-termini of the deduced chitinase sequence shows poor homology to chitinase II of *Aeromonas* sp., whereas internal sequence is relatively well conserved between the two enzymes. This may point to divergence in function of these regions, possibly related to export, since the *Aeromonas* enzyme has been shown to be exported via the general secretory pathway (Ueda *et al*., 1994).

The unusual structure seen downstream of the chitinase ORF could be related to termination of transcription, but the lack of polythimidine regions associated with the palindromic elements suggests that this is not typical of the *Rho* independent type (Carafa *et al*., 1990). Such a structure of direct repeats with nested inverted repeats could confer stability to the mRNA transcript, since the hairpin structures so formed are resistant to exoribonuclease attack. In other systems,

repetitive extragenic palindromic (REP) sequences have been associated with retro-regulation and mRNA processing (reviewed by Platt, 1986). These structures could function by causing RNA polymerase to pause, thereby forming a topological barrier to subsequent transcription or even possibly, translation (Dorman, 1994; Dorman, 1995). Another possibility is that this structure may be associated with the regulation of gene/s located downstream of *chi*A. Detailed functional analysis of this interesting region is therefore required to elucidate its possible involvement in chitinase regulation.

CHAPTER 7
General Conclusions

The study of mushroom diseases provides an excellent model for the interactions of microorganisms in a far from sterile environment. The problems encountered in these studies are very different to those encountered during the study of many animal diseases, where the resident microflora is either absent, in the case of sterile sites, or well understood. The nearest parallel is with studies on the diseases of plant roots etc., where the diseased tissues are in possible contact with the vast range of microorganisms encountered in soils. In this respect, much more work is required on the normal microflora of mushroom surfaces and tissues and on the susceptibilities of those tissues to microbial degradation. Where models exist, these are currently restricted to brown blotch disease caused by *P. tolaasii*. This is also the only system where we have significant data on the relative resistance to infection (or rather disease manifestation) of certain *A. bisporus* cultivars (Peng, 1986). Resistance to disease is, surprisingly, a currently neglected area and should be considered in breeding programmes alongside other factors such as yield and flavour. Progress does, however, appear to have been made in the discovery of more effective chemical controls for bacterial mushroom diseases (Wong & Preece, 1985; Geels, 1995), although these are dependent on market and legislative acceptability for such chemicals in foodstuffs. Biological disease control may be a viable alternative and has been used to experimentally control brown blotch with some success (Nair & Fahy, 1976; Fermor & Lynch, 1988). However, commercial

acceptance of this technology may be dependent on the development of more effective agents and on the premise that these agents offer economic advantages over the currently used chemical alternatives. A possible alternative strategy could be to fully control the microbiology of the mushroom fruiting process. Here, all the media used in mushroom culture (including casing) may be pasteurised, and fruiting initiated by artificial application of efficient helper bacteria. This may have the advantages of greater certainty and reliability in fruiting control, and in the suppression of disease-causing bacteria entering mushroom houses subsequent to fruit-body initiation.

The results presented in this thesis have shown that *E. americana* is the cause of ISN in *A. bisporus*. In addition, the mushroom derived strains have been shown to be identical in properties to a clinically derived strain. There is currently no data in the literature regarding the environmental source of this organism, except that it is associated with rare cases of human disease. The possibility exists, that *E. americana* forms part of the normal human microflora and it is contamination of the mushroom houses with bacteria, either directly from workers, or indirectly from sewage contamination of water supplies / media that results in their introduction. *E. americana* has, however, been found in the guts of *Anopheles stephensii* mosquitoes (Pumpuni *et al*., 1993), presumably forming part of the resident microflora. If this is a widespread phenomenon in other insects, then this could be a possible reservoir for these bacteria in nature. A connection may also be made with the chitinolytic properties of these bacteria, which may be important in the colonisation of an insect host. Additionally, the possibility of the possession of a chitin binding protein by

these bacteria may be involved in their attachment both to insect and to fungal surfaces.

The molecular fingerprint developed for *E. americana*, based on RFLPs, assisted greatly in the proof of Koch's postulates in this system, essentially allowing the differentiation of applied strains from endemic bacteria. Rapid and sensitive diagnostic technology could be developed in this system, involving PCR for the detection of *E. americana* in environmental samples and diseased mushrooms. A suitable target for the design of specific PCR primers could be the chitinase gene sequenced in this study. The *chi*A sequence contains regions unique (to date) to *E. americana.* Alignment data also suggests that localised variability is common among these genes in bacteria, meaning that such sequences should allow species differentiation. Such primers should, however, be shown to be able to detect the range of strains encountered, since in this study, RFLP data indicates significant polymorphism at the genetic level between strains, and in the case of ISN strain PI98, in the genomic vicinity of the *chi*A locus.

Chitinases have been shown to be a key factor in several biocontrol systems (Ordentlich *et al*., 1988; Chernin *et al*., 1995), and this type of antagonism may reflect an essential role in ISN pathogenesis. The characterisation of the chitinase of *E. americana*, and the cloning of its encoding gene provides an opportunity to experimentally dissect the functional role of this enzyme. Null mutations may be produced by insertion into the proximal regions of the *chiA* ORF of disrupting cassettes such as the Ω construct of Prentki & Krisch (1984), and mini-Tn5 transposon derivatives (De Lorenzo *et al*., 1990). Such constructs have the advantage of being directly selectable, by virtue of antibiotic resistance genes

forming part of the mutagenic fragment. The construct may then be introduced into the *E. americana* genome using a suitable mobilisation system, and integrated into the *chi*A locus by endogenous homologous recombination. Mutant strains may then be tested for pathogenicity against *A. bisporus*.

Similar techniques may be applied in the investigation of other potentially significant characteristics of *E. americana*, such as lipase, siderophore production and the possible production of a chitin-binding lectin. The latter character, once purified, may be investigated at a functional level by utilising the techniques of immunoelectron microscopy to localise the protein at the bacterial surface, or at the host / pathogen interface. More experimental evidence is also required regarding the properties of this character in the adhesion of *E. americana* to hyphae in the fruit bodies. A preliminary experiment could be to quantitatively investigate the possible inhibitory effect of chitooligosaccharides on the immobilisation of the bacteria to both an insoluble chitin support and an *A. bisporus* cell wall preparation.

E. americana is typical of the *Enterobacteriaceae* in being peritrichously flagellated and motile, which might be important in respect of the initial invasion of mushrooms at early stages in their development. Motility and flagella are clearly involved directly in the invasiveness of some organisms such as *Campylobacter jejuni* (Yao *et al*., 1994), where in addition to an essential role in taxis and invasion, the flagellin component of the flagellum was suggested as possibly acting as a secondary adhesin. The significance of flagella in pathogenicity may also be associated with protein secretion, as has been shown to be the case in *Erwinia carotovora* subspecies *atroseptica* (Mulholland *et al*., 1993). This situation may be mirrored in *E. americana*, where the 33 kDa chitinase appears to be synthesised

constitutively in the stationary growth phase and lacks a typical N-terminal signal sequence. Chitinase secretion, therefore, is presumably dependant on an alternative export mechanism, as appears to be the case with the chitinase B of *Serratia marcescens* BLJ200 (Brurberg *et al*., 1995). Several genes associated with flagellum export and assembly have been shown to show similarities to certain pathogenicity associated genes (Finan *et al*., 1995). In *Serratia liquefaciens*, for example, synthesis and secretion of phospholipase was found to be controlled by the master controlling gene of the flagellar operon (Givskov *et al*., 1995).

Current evidence indicates that the chitinase examined in this study is the only such enzyme possessed by *E. americana*. Other lines of evidence also suggest that this enzyme is not primarily involved in the utilisation of chitin as a source of carbon for growth. This supports the hypothesis that this enzyme may be implicated in the colonisation by *E. americana* of a living chitinaceous host, be that *A. bisporus*, or a currently unidentified natural reservoir. Furthermore, the molecular characteristics of the enzyme itself, suggests that it is not primarily suited for the environmental mineralisation of pre-formed chitin, but rather, for the breakdown of nascent chitin *in situ*. Considerable progress has recently been made on the crystallographic properties of certain chitinases. Multiple alignment and mutagenesis studies have also begun to clarify the significance of certain conserved motifs present in these enzymes. Deletion of conserved region 2 within the catalytic domain of the *E. americana* ChiA protein, previously implicated as forming the active site of chitinases, produced a product retaining significant chitinolytic activity. Further deletion analysis and site-directed mutagenesis may, therefore, clarify the role of the remaining highly conserved amino-acids of region 1. Such

evidence may eventually lead to a reappraisal of the significance of these motifs in catalysis. The *chiA* locus also demonstrates several interesting characteristics, possibly relating to the regulation of chitinase production. Detailed promoter mapping studies are required to elucidate possible activating sequences, which were not identified in this study simply by sequence analysis. A difficulty is the fact that this is the first gene to be sequenced in *E. americana*, meaning that we have no access to relevant consensus data. Similar comments may also be applied to the unusual structure of the region downstream of the *chiA* ORF. The *Enterobacteriaceae* are a relatively homogenous group in terms of general properties and rRNA homology, but gathering evidence suggests that at least at the level of gene organisation and regulation, considerable diversity is present. In the absence of experimental proof, therefore, we can make but few generalisations regarding the molecular genetics of these bacteria.

Appendix

Abbreviations for amino acids:

Amino acid	Three-letter abbreviation	One-letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionone	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

References

Akatsuka, H., Kawai, E., Omori, K., Komatsubara, S., Shibatani, T., Tosa, T. (1994) The *lipA* gene of *Serratia marcescens* which encodes an extracellular lipase has no N-terminal signal peptide. *Journal of Bacteriology* **176**, 1949-1956.

Allardet-Servent, A., Bouziges, N., Carles-Nurit, M.-J., Bourg, G., Gouby, A. & Ramuz, M. (1989) Use of low-frequency-cleavage restriction endonucleases for DNA analysis in epidemiological investigations of nosocomial bacterial infections. *Journal of Clinical Microbiology* **27**, 2057-2061.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990) Basic Local Alignment Search Tool. *Journal of Molecular Biology* **215**, 403-410.

Anon. (1989) API analytical profile index (API 20E). API system, S.A. Vercien, France. 3rd edition.

Anon. (1993) Promega Protein Guide: Tips and Techniques. Promega Corporation.

Arlorio, M., Ludwig, A., Boller, T. & Bonfante, P. (1992) Inhibition of fungal growth by plant chitinases and β-1-3-glucanases. *Protoplasma* **171**, 34-43.

Atkey, P. T., Fermor, T. R. & Lincoln, S. P. (1991) Bacterial soft rot of *Agaricus bitorquis* In: *Science and Cultivation of Edible fungi*. (Ed. M.J. Maher) pp.431-435. Balkema, Rotterdam.

Ayers, S. H., Rupp, P. & Johnson, W. T. (1919) A study of the alkali-forming bacteria in milk. *United States Department of Agriculture Bulletin* **782**

Bainton, N. J., Bycroft, B. W., Chhabra, S. R., Stead, P., Gledhill, L., Hill, P. J., Rees, C. E. D., Winson, M. K., Salmond, G. P. C., Stewart, G. S. A. B. & Williams,

P. (1992) A general role for the *lux* autoinducer in bacterial cell signalling: control of antibiotic synthesis in *Erwinia*. *Gene* **116**, 87-91.

Balasubramanian, R. & Manocha, M. S. (1992) Cytosolic and membrane-bound chitinases of two mucoraceous fungi: a comparative study. *Canadian Journal of Microbiology* **38**, 331-338.

Barrett, E. L., Solanes, R., E., Tang, J. S. & Palleroni, N. J. (1986) *Pseudomonas fluorescens* Boivar V: its resolution into distinct component groups and the relationship of these groups to other *P. fluorescens* Biovars, to *P. putida*, and to psychrotrophic pseudomonads associated with food spoilage. *Journal of General Microbiology* **132**, 2709-2721.

Bartnicki-Garcia, S. (1968) Cell wall chemistry, morphogenesis, and taxonomy of fungi. *Annual Review of Microbiology* **22**, 87-108.

Bawden, F. C. & Gregory, P. M. (1951) Mushroom mummy disease. *Rothamstead Experimental Station Annual Report* 84-85.

Beauchamp, C. J., Kloepper, J. W. & Lemke, P. A. (1993) Luminometric analyses of plant root colonisation by bioluminescent pseudomonads. *Canadian Journal of Microbiology* **39**, 434-441.

Beelman, R. B., Guthrie, B. D. & Royse, D. J. (1989). Influence of bacterial populations on postharvest deterioration of fresh mushrooms. In: *Mushroom Science XII (Part II): Proceedings of the Twelfth International Congress on the Science and Cultivation of Edible Fungi*, Braunschweig - Germany, 1987 pp.655-665.

Bernan, V., Filpula, D., Herber, W., Bibb, M. & Katz, E. (1985) The nucleotide sequence of the tyrosinase gene from *Streptomyces antibioticus* and characterisation of the gene product. *Gene* **37**, 101-110.

Bessette, A. E., Kerrigan, R. W. & Jordan, D. C. (1985) Yellow blotch of *Pleurotus ostreatus*. *Applied and Environmental Microbiology* **50**, 1535-1537.

Betterley, D. A. & Olson, J. A. (1989). Isolation, characterisation and studies of bacterial mummy disease of *Agaricus brunnescens*. In: *Mushroom Science XII (Part II): Proceedings of the Twelfth International Congress on the Science and Cultivation of Edible Fungi*, Braunschweig - Germany, 1987 pp.679-688.

Blaak, H., Schnellmann, J., Walter, S., Henrissat, B. & Schrempf, H. (1993) Characteristics of an exochitinase from *Streptomyces olivaceoviridis*, its corresponding gene, putative protein domains and relationship to other chitinases. *European Journal of Biochemistry* **214**, 659-669.

Bradford, M. M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein using the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248-254.

Brenner, D. J. (1978) Characterisation and clinical identification of *Enterobacteriaceae* by DNA hybridisation. *Progress in Clinical Pathology* **7**, 71-117.

Brodey, C. L., Rainey, P. B., Tester, M. & Johnstone, K. (1991) Bacterial blotch disease of the cultivated mushroom is caused by an ion channel forming lipodepsipeptide toxin. *Molecular Plant-Microbe Interactions* **4**, 407-411.

Brurberg, M. B., Eijsink, V. G. H., Haandrikman, A. J., Venema, G. & Nes, I. F. (1995) Chitinase B from *Serratia marcescens*-BJL200 is exported to the periplasm without processing. *Microbiology (UK)* **141**, 123-131.

Brurberg, M. B., Eijsink, V. G. H. & Nes, I. F. (1994) Characterisation of a chitinase gene (*chiA*) from *Serratia marcescens* BJL200 and one-step purification of the gene-product. *FEMS Microbiology Letters* **124**, 399-404.

Buchrieser, C., Brosch, R., Catimel, B. & Rocourt, J. (1993) Pulsed-field gel electrophoresis applied for comparing *Listeria monocytogenes* strains involved in outbreaks. *Canadian Journal of Microbiology* **39**, 395-401.

Bull, A. T. (1970) Inhibition of polysaccharases by melanin: enzyme inhibition in relation to mycolysis. *Archives of Biochemistry* **137**, 345-346.

Burton, K. S., Love, M. E. & Smith, J. F. (1993) Biochemical changes associated with mushroom quality in *Agaricus* spp. *Enzyme and Microbial Technology* **15**, 736-741.

Cabib, E. (1987) The synthesis and degradation of chitin. *Advances in Enzymology* **59**, 59-101.

Cammarano, P., Palm, P. & Creti, R. (1992) Early evolutionary relationships among known life forms inferred from elongation factor EF-2/EF-G sequences: phylogenetic coherence and structure of the archael domain. *Journal of Molecular Evolution* **34**, 396-405.

Carafa, Y.A., Brody, E., Thermes, C. (1990) Prediction of Rho-independent *Escherichia coli* transcription terminators. *Journal of Molecular Biology* **216**, 835-858.

Centron Garcia, D., Ruiz Trevisan, A., Botto, L., Cervetto, M., Sarubbi, M. A. & Zorzópulos, J. (1989) An outbreak of multiply resistant *Pseudomonas aeruginosa* in a neonatal unit: plasmid pattern analysis. *Journal of Hospital Infection* **14**, 99-105.

Champion, A. B., Barrett, E. L., Palleroni, N. J., Soderberg, K. L., Kunisawa, R., Contopoulou, R., Wilson, A. C. & Doudoroff, M. (1980) Evolution in *Pseudomonas fluorescens*. *Journal of General Microbiology* **120**, 485-511.

Chang, S.-T. (1993). Mushroom Biology: The impact on mushroom production and mushroom products. In: *First International conference on Mushroom Biology and Mushroom Products*. (Eds. S.-T. Chang, J.A. Buswell & S.-W. Chiu). pp.3-20, Chinese University Press, Hong Kong.

Chernin, L., Ismailov, Z., Haran, S. & Chet, I. (1995) Chitinolytic *Enterobacter agglomerans* antagonistic to fungal plant pathogens. *Applied and Environmental Microbiology* **61**, 1720-1726.

Chet, I., Barak, Z. & Oppenheim, A. (1993) Genetic engineering of microorganisms for improved biocontrol activity In: *Biotechnology in Plant Disease Control*. Wiley-Liss, pp.211-235.

Cole, A. L. J. & Skellerup, M. V. (1986) Ultrastructure of the interaction of *Agaricus bisporus* and *Pseudomonas tolaasii*. *Transactions of the British Mycological Society* **87**, 314-316.

Cooper, V. J. C. & Salmond, G. P. C. (1993) Molecular analysis of the major cellulase (*cel*V) of *Erwinia carotovora* - evidence for an evolutionary mix and match of enzyme domains. *Molecular and General Genetics* **241**, 341-350.

Craig, G. D., Wood, D. A. & Gull, K. (1981) Chitin synthase in the stipe of *Agaricus bisporus* (J. Lange) Imbach. *FEMS Microbiology Letters* **10**, 43-48.

Cutri, S. S., Macauley, B. J. & Roberts, W. P. (1984) Characteristics of pathogenic non-fluorescent (smooth) and non-pathogenic fluorescent (rough) forms of *Pseudmonas tolaasii* and *Pseudomonas gingeri*. *Journal of Applied Bacteriology* **57**, 291-298.

Danell, E., Alstrom, S. & Ternstrom, A. (1993) *Pseudomonas fluorescens* in association with fruit bodies of the ectomycorrhizal mushroom *Cantharellus cibarius*. *Mycological Research* **97**, 1148-1152.

Dauga, C., Grimont, F. & Grimont, P. A. D. (1990) Nucleotide sequences of 16S ribosomal RNA from 10 *Serratia* species. *Research in Microbiology* **141**, 1139-1149.

Deane, E. E. (1994) *Trichoderma harzianum* chitinase: biochemical, molecular and biocontrol properties. PhD Thesis, University of Nottingham.

De Lorenzo, V., Herrero, M., Jakubzik, U. & Timmis, K. N. (1990) Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in Gram-negative Eubacteria. *Journal of Bacteriology* **172**, 6568-6572.

Delic, I., Robbins, P. & Westpheling, J. (1992) Direct repeat sequences are implicated in the regulation of two *streptomyces* chitinase promoters that are subject to carbon catabolite control. *Proceedings of the National Academy of Science USA* **89**, 1885-1889.

Devreese, K., Claeys, G. & Verschraegen, G. (1992) Septicemia with *Ewingella americana*. *Journal of Clinical Microbiology* **30**, 2746-2747.

Doores, S., Kramer, M. & Beelman, R. (1986). Evaluation and bacterial populations associated with fresh mushrooms (*Agaricus bisporus*). In: *Proceedings of the International Symposium on the Scientific and Technical Aspects of Cultivating Edible Fungi*. (Eds. P.J. Weust, D.J. Royse & R.B. Beelman) pp.283-294. The Pennsylvania State University, PA. USA.

Dorman, C. J. (1994) *Genetics of Bacterial Virulence.* Blackwell Scientific Publications. Oxford. 1st edition.

Dorman, C. J. (1995) DNA topology and the global control of bacterial gene expression: implications for the regulation of virulence gene expression. *Microbiology (UK)* **141**, 1271-1280.

Dye, D. W., Bradbury, J. F., Dickey, R. S., Goto, M. S., Hale, C. N., Hayward, A. C., Kelman, A., Lelliott, R. A., Patel, P. N., Sands, D. S., Schroth, M. N., Watson, D. R. W. & Young, J. M. (1975) Proposals for a reappraisal of the status of names of plant-pathogenic *Pseudomonas* species. *International Journal of Systematic Bacteriology* **25**, 252-257.

Engwilmot, D. L., Adjimani, J. P. & Vanderhelm, D. (1992) Siderophore-mediated iron (III) transport in the mycelia of the cultivated fungus, *Agaricus bisporus*. *Journal of Inorganic Biochemistry* **48**, 183-195.

Ewing, W. H. (1986) *Edwards and Ewing's Identification of Enterobacteriaceae*. Elsevier. New York. 4th edition.

Falkow, S. (1988) Molecular Koch's postulates applied to microbial pathogenicity. *Reviews in Infectious Diseases* **10**, S274-S276.

Farmer III, J. J., Davis, B. R., Hickman-Brenner, F. W., McWhorter, A., Huntley-Carter, G. P., Asbury, M. A., Riddle, C., Wathen-Grady, H. G., Elias, C., Fanning, G. R., Steigerwalt, A. G., O'Hara, C. M., Morris, G. K., Smith, P. B. & Brenner, D. J. (1985) Biochemical identification of new species and biogroups of *Enterobacteriaceae* isolated from clinical specimens. *Journal of Clinical Microbiology* **21**, 46-76.

Fermor, T. R., Randle, P. E. & Smith, J. F. (1985) Compost as a substrate and its preparation In: *The biology and technology of the cultivated mushroom*. (Eds. P.B. Flegg, D.M. Spencer & D.A. Wood) pp.81-109. Wiley, Chichester.

Fermor, T. R. (1986). Bacterial diseases of edible mushrooms and their control. In: *Proceedings of the International Symposium on the Scientific and Technical Aspects of Cultivating Edible Fungi*. (Eds. P.J. Weust, D.J. Royse & R.B. Beelman) pp.361-369. The Pennsylvania State University, PA. USA.

Fermor, T. R. & Lynch, J. M. (1988) Biological control of bacterial blotch disease (*Pseudomonas tolaasii*) of the cultivated mushroom *Agaricus bisporus*: screening, isolation and characterisation of bacteria antagonistic to the pathogen. *Journal of Applied Bacteriology* **65**, 179-187.

Fermor, T. R. & Wood, D. A. (1991) Mushroom compost microbial biomass: A review. In: *Science and Cultivation of Edible fungi*. (Ed. M.J. Maher) pp.191-199. Balkema, Rotterdam.

Fermor, T. R., Wood, D. A., Lincoln, S. P. & Fenlon, J. S. (1991) Bacteriolysis by *Agaricus bisporus*. *Journal of General Microbiology* **137**, 15-22.

Finan, T. M., Gough, C. & Truchet, G. (1995) Similarity between the *Rhizobium meliloti fliP* gene and pathogenicity associated genes from animal and plant pathogens. *Gene* **152**, 65-67.

Flach, J., Pilet, P. E. & Jollès, P. (1992) What's new in chitinase research? *Experientia* **48**, 701-716.

Fletcher, J. T., White, P. F. & Gaze, R. H. (1989) *Mushrooms: pest and disease control.* Intercept. Andover, Hants. 2nd edition.

Flurkey, W. H. & Ingebrigtsen, J. (1989) Polyphenol oxidase activity and enzymatic browning in mushrooms. In: *Quality factors in fruit and vegetables*. American Chemical Society, pp.44-54.

Fox, G. E. & Stackebrandt, E. (1987) The application of 16S rRNA cataloguing and 5S rRNA sequencing in bacterial systematics. *Methods in Microbiology* **19**, 405-458.

Fox, G. E., Wisotzkey, J. D. & Jurtshuk, P. (1992) How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *International Journal of Systematic Bacteriology* **42**, 166-170.

Frändberg, E. & Schnürer, J. (1994) Evaluation of a chromogenic chito-oligosaccharide analog, *p*-nitrophenyl-β-D-*N,N'*-diacetylchitobiose, for the measurement of the chitinolytic activity of bacteria. *Journal of Applied Bacteriology* **76**, 259-263.

Freeman, R., Goodfellow, M., Ward, A. C., Hudson, S. J., Gould, F. K. & Lightfoot, N. F. (1991) Epidemiological typing of coagulase-negative staphylococci by pyrolysis mass spectrometry. *Journal of Medical Microbiology* **34**, 245-248.

Fuchs, R. L., McPherson, S. A. & Drahos, D. J. (1986) Cloning of a *Serratia marcescens* gene encoding chitinase. *Applied and Environmental Microbiology* **51**, 504-509.

Fuji, T. & Miyashita, K. (1993) Multiple domain structure in a chitinase gene (*chiC*) of *Streptomyces lividans*. *Journal of General Microbiology* **139**, 677-686.

Gandy, D. G. (1968) A technique for screening bacteria causing brown blotch of cultivated mushrooms. *Annual Report of the Glasshouse Crops Research Institute for 1967* 150-156.

Garcia Mendoza, C., Avellan, M. A., Sanchez, E. & Novaes-Ledieu, M. (1987) Differentiation and wall chemistry of *Agaricus bisporus* vegetative and aggregated mycelia. *Archives of Microbiology* **148**, 68-71.

Gavini, F., Mergaert, J., Beji, A., Mielcarek, C., Izard, D., Kersters, K. & De Ley, J. (1989) Transfer of *Enterobacter agglomerans* (Beijerinck 1888) Ewing and Fife 1972 to *Pantoea agglomerans* comb. nov. and description of *Pantoea dispersa* sp. nov. *International Journal of Systematic Bacteriology* **39**, 337-345.

Geels, F. P. (1995) *Pseudomonas tolaasii* control by kasugamycin in cultivated mushrooms (*Agaricus bisporus*). *Journal of Applied Bacteriology* **79**, 38-42.

Geels, F. P., Griensven, L. J. L. D. & Rutjens, A. J. (1991) Chlorine dioxide and the control of bacterial blotch on mushrooms caused by *Pseudomonas tolaasii* In: *Science and Cultivation of Edible Fungi*. (Ed. M.J. Maher) pp.437-442. Balkema, Rotterdam.

Geels, F. P., Hesen, L. P. W. & Vangriensven, L. J. L. D. (1994) Brown discoloration of mushrooms caused by *Pseudomonas agarici*. *Journal of Phytopathology- Phytopathologische Zeitschrift* **140**, 249-259.

Gennari, M. & Dragotto, F. (1992) A study of the incedence of different fluorescent *Pseudomonas* species and biovars in the microflora of fresh and spoiled meat and fish, raw milk, chese, soil and water. *Journal of Applied Bacteriology* **72**, 281-288.

Gilkes, N. R., Henrissat, B., Kilburn, D. G., Miller, R. C. J. & Warren, R. A. J. (1991) Domains in microbial β-1,4-glycanases: sequence conservation, function and enzyme families. *Microbiological Reviews* **55**, 303-315.

Gill, W. M. & Cole, A. L. J. (1992) Cavity disease of *Agaricus bisporus* caused by *Pseudomonas cepacia*. *Canadian Journal of Microbiology* **38**, 394-397.

Givskov, M., Eberl, L., Christiansen, G., Bebedik, M. J. & Molin, S. (1995) Induction of phospholipase and flagellar synthesis in *Serratia liquefaciens* is controlled by expression of the flagellar master operon *flhD*. *Molecular Microbiology* **15**, 445-454.

Gleave, A. P., Taylor, R. K., Morris, B. A. & Greenwood, D. R. (1995) Cloning and sequencing of a gene encoding the 69 kDa extracellular chitinase of *Janthinobacterium lividum*. *FEMS Microbiology Letters* **131**, 279-288.

Gold, L., Pribnow, D., Schneider, T., Shinedling, S., Singer, B. S. & Stormo, G. (1981) Translation initiation in prokaryotes. *Annual Review of Microbiology* **35**, 365-403.

Goldmann, D. A. & Klinger, J. D. (1986) *Pseudomonas cepacia*: biology, mechanisms of virulence, epidemiology. *Journal of Paediatrics* **108**, 806-812.

Grant, F. A., Glover, L. A., Killham, K. & Prosser, J. I. (1991) Luminescence-based viable cell enumeration of *Erwinia carotovora* in the soil. *Soil Biology and Biochemistry* **23**, 1021-1024.

Grewal, S. I. S. & Rainey, P. B. (1991) Phenotypic variation of *Pseudomonas putida* and *P. tolaasii* affects the chemotactic response to *Agaricus bisporus* mycelium. *Journal of General Microbiology* **137**, 2761-2768.

Grewal, S. I. S., Han, B. & Johnstone, K. (1994) Regulation of toxin synthesis and phenotypic variation in *Pseudomonas tolaasii*, cause of brown blotch disease of mushrooms In: *Advances in molecular genetics of plant-microbe interactions*. (Eds. M.J. Daniels) pp.25-32. Kluwer Academic Publishers, Amsterdam.

Grimont, P. A. D., Grimont, F. & Starr, M. P. (1979) *Serratia proteamaculans* (Paine and Stansfield) comb. nov., a senior subjective synonym of *Serratia liquefaciens* (Grimes and Hennerty) Bascomb *et al*. *International Journal of Systematic Bacteriology* **28**, 503-510.

Grimont, P. A. D., Popoff, M. Y., Grimont, F., Coynault, C. & Lemelin, M. (1980) Reproducibility and correlation study of three deoxyribonucleic acid hybridisation procedures. *Current Microbiology* **4**, 325-330.

Grimont, P. A. D., Farmer III, J. J., Grimont, F., Asbury, M. A., Brenner, D. J. & Deval, C. (1983) *Ewingella americana* Gen.Nov., Sp. Nov., A new

Enterobacteriaceae isolated from clinical specimens. *Annals of Microbiology (Institut Pasteur)* **134**, 39-52.

Grimont, F. & Grimont, P. A. D. (1986) Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. *Annals of the Institut Pasteur Microbiology* **137B**, 165-175.

Gross, R. J. & Holmes, B. (1990) The *Enterobacteriaceae* In: *Topley & Wilson's Principles of bacteriology, virology and immunity*. (Eds. M.T. Parker & B.I. Duerden) pp.404-436. Edward Arnold, London.

Grothues, D. & Tümmler, B. (1987) Genome analysis of *Pseudomonas aeruginosa* by field inversion gel electrophoresis. *FEMS Microbiology Letters* **48**, 419-422.

Gulig, P. A. (1993) Use of isogenic mutants to syudy bacterial virulence factors. *Journal of Microbiological Methods* **18**, 275-287.

Gutell, R. R. (1993) Collection of small subunit (16S- and 16S-like) ribosomal RNA structures. *Nucleic Acids Research* **21**, 3051-3054.

Gutell, R. R., Larsen, N. & Woese, C. R. (1994) Lessons from an evolving rRNA: 16S and 23S rRNA structures from a comparative perspective. *Microbiological Reviews* **58**, 10-26.

Guthrie, B. D. & Beelman, R. B. (1989). Control of bacterial deterioration in fresh washed mushrooms. In: *Mushroom Science XII (Part II): Proceedings of the Twelfth International Congress on the Science and Cultivation of Edible Fungi*, Braunschweig - Germany, 1987. pp.689-699.

Hall, L. M. C. (1994) Are point mutations or DNA rearrangements responsible for the restriction fragment length polymorphisms that are used to type bacteria? *Microbiology* **140**, 197-204.

Hans, F. S., Mortishire-Smith, R. J., Rainey, P. B. & Williams, D. H. (1992) Structure of the white-line inducing principle isolated from *Pseudomonas reactans*.

Acta Crystallographica; Section C- Crystal Structure Communications **48**, 1965-1968.

Haran, S., Schickler, H., Oppenheim, A. & Chet, I. (1995) New components of the chitinolytic system of *Trichoderma harzianum*. *Mycological Research* **99**, 441-446.

Hartstein, A. I., Morthland, V. H., Eng, S., Archer, G. L., Schoenknecht, F. D. & Rashad, A. L. (1989) Restriction enzyme analysis of plasmid DNA and bacteriophage typing of paired *Staphylococcus aureus* blood culture isolates. *Journal of Clinical Microbiology* **27**, 1874-1879.

Hayes, W. A., Randle, P. E. & Last, F. T. (1969) The nature of the microbiological stimulus affecting sporophore formation in *Agaricus bisporus*. *Annals of Applied Biology* **64**, 177-187.

Hearing, V. J. (1987) Mammalian monophenol monooxygenase (Tyrosinase): Purification, properties, and reactions catalyzed. *Methods in Enzymology* **142**, 154-164.

Hedrick, S. A., Bell, J. N., Boller, T. & Lamb, C. J. (1988) Chitinase cDNA cloning and mRNA induction by fungal elicitor, wounding and infection. *Plant Physiology* **86**, 182-186.

Hegnauer, H., Nylen, L. E. & Rast, D. M. (1985) Ultrastructure of native and synthetic *Agaricus bisporus* melanins - Implications as to compartmentation of melanogenesis in fungi. *Experimental Mycology* **9**, 221-229.

Held, T. & Kutzner, H. J. (1990) Transcription of the tyrosinase gene in *Streptomyces michiganensis* DSM 40015 is induced by copper and repressed by ammonium. *Journal of General Microbiology* **136**, 2413-2419.

Henikoff, S. (1984) Unidirectional digestion with exonuclease creates targeted breakpoints for DNA sequencing. *Gene* **28**, 357.

Henrissat, B. (1990) Weak sequence homologies among chitinases detected by clustering analysis. *Protein Sequences & Data Analysis* **3**, 523-526.

Henry, M. B., Lynch, J. M. & Fermor, T. R. (1991) Role of siderophores in the biocontrol of *Pseudomonas tolaasii* by fluorescent pseudomonad antagonists. *Journal of Applied Bacteriology* **70**, 104-108.

Hirsch, H. M. (1954) Environmental factors influencing the differentiation of protoperithecia and their relation to tyrosinase and melanin formation in *Neurospora crassa*. *Physiologia Plantarum* **7**, 72-97.

Hobohm, U., Houthaeve, T. & Sander, C. (1994) Amino acid analysis and protein database composition search as a fast and inexpensive method to identify proteins. *Analytical Biochemistry* **222**, 202.

Holloway, B. W., Escuadra, M. D., Morgan, A. F., Saffery, R. & Krishnapillai, V. (1992) The new approaches to whole genome analysis of bacteria. *FEMS Microbiology Letters* **100**, 101-106.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T. & Williams, S. T. (1994) *Bergey's Manual of Determinative Bacteriology.* Williams & Wilkins. Baltimore. 9th edition.

Horowitz, N. H., Fling, M., Macleod, H. & Watanabe, Y. (1960) Genetic determination and enzymic induction of tyrosinase in *Neurospora*. *Journal of Molecular Biology* **2**, 96-104.

Huber, M., Hinterman, G. & Lerch, K. (1985) Primary structure of tyrosinase from *Streptomyces glaucescens*. *Biochemistry* **24**, 6038-6044.

Hutchison, M. L. & Johnstone, K. (1993) Evidence for the involvement of the surface activity of the extracellular toxin tolaasin in the manifestation of brown blotch disease symptoms by *Pseudomonas tolaasii* on *Agaricus bisporus*. *Physiological and Molecular Plant Pathology* **42**, 373-384.

Ikeda, S., Toyoda, H., Matsuda, Y. & Ouchi, S. (1995) DNA sequence determination of a chitinase gene *chiSHI* cloned from the Gram-positive bacterium,

Kurthia zopfii and its application to biological control of powdery mildew of barley. *Unpublished Genbank Submission*

Ilyina, A. V., Varlamov, V. P., Tikhonov, V. E., Yamskov, I. A. & Davankov, V. A. (1994) One-step isolation of a chitinase by affinity chromatography of the chitinolytic enzyme complex produced by *Streptomyces kurssanovii*. *Biotechnology and Applied Biochemistry* **19**, 199-207.

Jahnke, K. D. (1994) A modified method of quantitative colorimetric DNA-DNA hybridisation on membrane filters for bacterial identification. *Journal of Microbiological Methods* **20**, 273-288.

Jones, J. B., Chase, A. R. & Harris, G. K. (1993) Evaluation of the Biolog GN microplate system for identification of some plant-pathogenic bacteria. *Plant Disease* **77**, 553-558.

Jones, J. D. G., Grady, K. L., Suslow, T. V. & Bedbrook, J. (1986) Isolation and characterisation of genes encoding two chitinase enzymes from *Serratia marcescens*. *The EMBO Journal* **5**, 467-473.

Jones, J. D. G., Dean, C., Gidoni, D., Gilbert, D., Bond-Nutter, D., Lee, R., Bedbrook, J. & Dunsmuir, P. (1988) Expression of bacterial chitinase protein in tobacco leaves using two photosynthetic gene promoters. *Molecular and General Genetics* **212**, 536-542.

Jordens, J. Z. (1991) Restriction enzyme analysis of chromosomal DNA and its application in epidemiological studies. *Journal of Hospital Infection* **18**, 432-437.

Joshi, S., Kozlowski, M., Selvaraj, G., Iyer, V. N. & Davies, R. W. (1988) Cloning of the genes of the chitin utilization regulon of *Serratia liquefaciens*. *Journal of Bacteriology* **170**, 2984-2988.

Kamei, K., Yamamura, Y., Hara, S. & Ikenaka, T. (1989) Amino acid sequence of chitinase from *Streptomyces erythraeus*. *Journal of Biochemistry* **105**, 979-985.

Katz, E., Thompson, C. J. & Hopwood, D. A. (1983) Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. *Journal of General Microbiology* **129**, 2703-2714.

Kenneth, H. (1995) Molecular biology as a tool for taxonomy. *Clinical Infectious Diseases* **20**, s 117-121.

King, E. O., Ward, M. K. & Raney, D. E. (1954) Two simple media for the demonstration of pyocyanin and fluorescein. *Journal of Laboratory and Clinical Medicine* **44**, 301-307.

Kless, H., Sitrit, Y., Chet, I. & Oppenheim, A. B. (1989) Cloning of the gene coding for chitobiase of *Serratia marcescens*. *Molecular and General Genetics* **217**, 471-473.

Kupper, U., Linden, M., Cao, K. & Lerch, K. (1990) Expression of tyrosinase in vegetative cultures of *Neurospora crassa* transformed with a metallothionein promoter/protyrosinase fusion gene. *Current Genetics* **18**, 331-335.

Kuranda, M. J. & Robbins, P. W. (1991) Chitinase is required for cell separation during growth of *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **266**, 19758-19767.

Kurtzman, C. P. (1993) DNA-DNA hybridisation approaches to species identification in small genome organisms In: *Molecular Evolution: Producing the Biochemical Data*. E.A. Zimmer, T.J. White, R.L. Cann & A.C. Wilson (Eds.) Academic Press, San Diego. pp.335-348.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of bacteriophage T4. *Nature* **227**, 680-683.

Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L. & Pace, N. R. (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proceedings of the National Academy of Science USA* **82**, 6955-6959.

Lelliot, R. A. & Stead, D. E. (1987) *Methods for the diagnosis of bacterial diseases of plants*. Blackwell. Oxford.

Lin, T.-Y. & Koshland, D. E. J. (1969) Carboxyl group modification and the activity of lysozyme. *Journal of Biological Chemistry* **244**, 505-508.

Lincoln, S. P., Fermor, T. R., Stead, D. E. & Sellwood, J. E. (1991) Bacterial soft rot of *Agaricus bitorquis*. *Plant Pathology* **40**, 136-144.

Loewen, P. C. & Hengge-Aronis, R. (1994) The role of the sigma factor σS (KatF) in bacterial global regulation. *Annual Review of Microbiology* **48**, 53-80.

Lorito, M., Harman, G. E., Hayes, C. K., Broardway, R. M., Tronsmo, A., Woo, S. L. & Di Pietro, A. (1993b) Chitinolytic enzymes produced by *Trichoderma harzianum*: antifungal activity of purified endochitinase and chitobiosidase. *Phytopathology* **83**, 302-307.

Luisetti, J., Prunier, J. P. & Gardan, L. (1972) Un milieu pour la mise en évidence de la production d'un pigment fluorescent par *Pseudomonas mors-prunorum* f.sp. *persicae*. *Annals of Phytopathology* **4**, 295-296.

Lund, P. & Dunsmuir, P. (1992) A plant signal sequence enhances the secretion of bacterial ChiA in transgenic tobacco. *Plant Molecular Biology* **18**, 47-53.

MacConkey, A. T. (1908) Bile salt media and their advantages in some bacteriological examinations. *Journal of Hygiene, Cambridge* **8**, 322.

Maddaluno, J. F. & Faull, K. F. (1988) Inhibition of mushroom tyrosinase by 3-amino-L-Tyrosine - molecular probing of the active site of the enzyme. *Experientia* **44**, 885-887.

Malcolm, B. A., Rosenberg, S., Correy, M. J., Allen, J. S., de Baetselier, A. & Kirsch, J. F. (1989) Site-directed mutagenesis of the catalytic residues Asp-52 and Glu-35 of chicken egg white lysozyme. *Proceedings of the National Academy of Sciences USA* **86**, 133-137.

Margis-Pinheiro, M., Metz-Boutigue, M. H., Awade, A., de Tapia, M., le Ret, M. & Burkard, G. (1991) Isolation of a complementary DNA encoding the bean PR4 chitinase: an acidic enzyme with an amino-terminus cysteine-rich domain. *Plant Molecular Biology* **17**, 243-253.

Masson, J.-Y., Denis, F. & Brzezinski, R. (1994) Primary sequence of the chitosanase from *Streptomyces* sp. strain N174 and comparison with other endoglycosidases. *Gene* **140**, 103-107.

Mayer, A. M. & Harel, E. (1979) Polyphenoloxidases in plants. *Phytochemistry* **18**, 193-215.

McClelland, M., Jones, R., Patel, Y. & Nelson, M. (1987) Restriction endonucleases for pulsed-field mapping of bacterial genomes. *Nucleic Acids Research* **15**, 5985-6005.

McCreath, K. J. & Gooday, G. W. (1992) A rapid and sensitive microassay for determination of chitinolytic activity. *Journal of Microbiological Methods* **14**, 229-237.

Metraux, J. P., Burkhart, W., Moyer, M., Dincher, S., Middlesteadt, W., Williams, S., Payne, G., Carnes, M. & Ryales, J. (1989) Isolation of a complementary DNA encoding a chitinase with structural homology to a bifunctional lysozyme/chitinase. *Proceedings of the National Academy of Sciences USA* **86**, 896-900.

Milewski, S., O'Donnell, R. W. & Gooday, G. W. (1992) Chemical modification studies of the active centre of *Candida albicans* chitinase and its inhibition by allosamidin. *Journal of General Microbiology* **138**, 2545-2550.

Miller, J. H. (1972) *Experiments in Molecular Genetics.* Cold Spring Harbor Laboratory. Cold Spring Harbor, NY.

Minke, J. L. & Blackwell, J. (1978) The structure of α chitin. *Journal of Molecular Biology* **120**, 167-181.

Miyashita, K., Fujii, T. & Sawada, Y. (1991) Molecular cloning and characterisation of chitinase genes from *Streptomyces lividans* 66. *Journal of General Microbiology* **137**, 2065-2072.

Mol, P. C., Vermeulen, C. A. & Wessels, J. G. H. (1990) Diffuse extension of hyphae in stipes of *Agaricus bisporus* may be based on a unique wall structure. *Mycological Research* **94**, 480-488.

Mol, P. C. & Wessels, J. G. H. (1990) Differences in wall structure between substrate hyphae and hyphae of fruit-body stipes in *Agaricus bisporus*. *Mycological Research* **94**, 472-479.

Molano, J., Polacheck, U., Duran, A. & Cabib, E. (1979) An endochitinase from wheat germ. Activity on nascent and preformed chitin. *Journal of Biological Chemistry* **254**, 4901-4907.

Montgomery, M. T. & Kirchman, D. L. (1993) Role of chitin-binding proteins in the specific attachment of the marine bacterium *Vibrio harveyi* to chitin. *Applied and Environmental Microbiology* **59**, 373-379.

Moore, B. M., Kang, B. & Flurkey, W. H. (1988) Histochemical and Immunochemical localisation of tyrosinase in whole tissue sections of mushrooms. *Phytochemistry* **27**, 3735-3737.

Morrisey, R. F., Dugan, E. P. & Koths, J. S. (1976) Chitinase production by an *Arthrobacter* sp. lysing cells of *Fusarium roseum*. *Soil Biology and Boichemistry* **8**, 23-28.

Morrison, R., Mason, K. & Frostmason, S. (1994) A cladistic analysis of the evolutionary relationships of the members of the tyrosinase gene family using sequence data. *Pigment Cell Research* **7**, 388-393.

Mortishire-Smith, R. J., Drake, A. F., Nutkins, J. C. & Williams, D. H. (1991) Left-handed α-helix formation by a bacterial peptide. *FEBS Letters* **278**, 244-246.

Mukwaya, G. M. & Welch, D. F. (1989) Subgrouping of *Pseudomonas cepacia* by cellular fatty acid composition. *Journal of Clinical Microbiology* 2640-2646.

Mulholland, V., Hinton, J. C. D., Sidebotham, J., Toth, I. K., Hyman, L. J., Perombelon, M. C. M., Reeves, P. J. & Salmond, G. P. C. (1993) A pleiotropic reduced virulence (RVI-) mutant of *Erwinia carotovora* subspecies atroseptica is defective in flagella assembly proteins that are conserved in plant and animal bacterial pathogens. *Molecular Microbiology* **9**, 343-356.

Munjal, A., Khanna, P. K. & Garcha, H. S. (1987). *In vitro* chemical and biological control of bacterial blotch of *Agaricus bisporus* (Sing.) Lange. In: *Mushroom Science XII (Part II): Proceedings of the Twelfth International Congress on the Science and Cultivation of Edible Fungi*, Braunschweig - Germany, 1987 pp.667-677.

Muzzarelli, R. A. A. (1988) Carboxymethylated chitins and chitosans. *Carbohydrate Polymers* **8**, 1-21.

Nair, N. G. & Fahy, P. C. (1972) Bacteria antagonistic to *Pseudomonas tolaasii* and their control of brown blotch of the cultivated mushroom, *Agaricus bisporus*. *Journal of Applied Bacteriology* **35**, 439-442.

Nair, N. G. & Fahy, P. C. (1973) Toxin production by *Pseudomonas tolaasii*. *Australian Journal of Biological Science* **26**, 509-512.

Nair, N. G. & Fahy, P. C. (1976) Commercial application of biological control of mushroom bacterial blotch. *Australian Journal of Agricultural Research* **27**, 415-422.

Nakahama, K., Yoshimura, K., Marumoto, R., Kikuchi, M., Lee, I.S., Hase, T., Matsubara, H. (1986) Cloning and sequencing of *Serratia* protease gene. *Nucleic Acids Research* **14**, 5843-5855.

Nakai, Y., Ushiyama, R. & Komatsu, M. (1982) Presence of a rod-shaped bacterium in *Lentinus edodes* fruit bodies with a browning symptom. *Report of Tottori Mycological Institute (Japan)* **20**, 47-53.

Nakai, Y. & Ushiyama, R. (1985) Intracellular appearance of a rickettsia-like organism in *Lentinus edodes*. *Report of the Tottori Mycological Institute (Japan)* **22**, 84-85.

Noble, W. C. & Rahman, M. (1986) Plasmid profiling of epidemic staphylococci from around 1960: a comparison of epidemiologic techniques. *Journal of Hygiene* **97**, 211-218.

Nutkins, J. C., Mortishire-Smith, R. J. & Packman, L. C. (1991) Structure determination of tolaasin, an extracellular lipodepsipeptide produced by the mushroom pathogen *Pseudomonas tolaasii* Paine. *Journal of the American Chemical Society* **113**, 2621-2627.

O'Donoghue-Maguire, D. C. & Ryan, J. P. (1991) Influences of a wide range of bacteria, actinomycetes and fungi on mycelial growth of *Agaricus bisporus* (Lange), Sing and the special fruiting requirements of *A. bisporus* In: *Science and Cultivation of Edible Fungi*. (Ed. M.J. Maher) pp.753-759. Balkema, Rotterdam.

Oedjijono, Line, M. A. & Dragar, C. (1993) Isolation of bacteria antagonistic to plant-pathogenic fungi. *Soil Biology & Biochemistry* **25**, 247-250.

Ohtakara, A. (1988) Viscosimetric assay for chitinase. *Methods in Enzymology* **161**, 426-430.

Oliver, J. M., Guillaumes, J. & Martin, D. (1978). Study of bacterial disease of mushroom caps. In: *Proceedings of the 4th International Conference of Plant Pathogenic Bacteria*, Angers, France. S.d.P.V. Phytobacteriologie, pp.903-916.

Ordentlich, A., Elad, Y. & Chet, I. (1988) The role of chitinase of *Serratia marcescens* in biocontrol of *Sclerotium rolfsii*. *Phytopathology* **78**, 84-87.

Owen, R. J. (1989) Chromosomal DNA fingerprinting - a new method of species and strain identification applicable to microbial pathogens. *Journal of Medical Microbiology* **30**, 89-99.

Oxley, S. J. P. (1984) Etiology and epidemiology of mummy disease of the cultivated mushroom *Agaricus bisporus*. PhD Thesis, University of Leeds.

Paget, M. S. B., Hintermann, G. & Smith, C. P. (1994) Construction and application of streptomycete promoter probe vectors which employ the *Streptomyces glaucescens* tyrosinase encoding gene as reporter. *Gene* **146**, 105-110.

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

Palleroni, N. J. (1984) Genus I. *Pseudomonas* Migula 1894 In: *Bergey's Manual of Systematic Bacteriology*. (Eds. N.R. Krieg & J.G. Holt) pp.141-219. Williams and Wilkins, Baltimore.

Palleroni, N. J. (1993) *Pseudomonas* classification - a new case-history in the taxonomy of Gram-negative bacteria. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **64**, 231-251.

Palleroni, N. J. & Doudoroff, M. (1972) Some properties and taxonomic sub-divisions of the genus *Pseudomonas*. *Annual Review of Phytopathology* **10**, 73-100.

Palleroni, N. J., Kunisawa, R., Contopoulou, R. & Doudoroff, M. (1973) Nucleic acid homologies in genus *Pseudomonas*. *International Journal of Systematic Bacteriology* **23**, 333-339.

Pearson, W. R. & Lipman, D. J. (1988) Improved tools for biological sequence comparison. *Proceedings of the National Academy of Sciences USA* **85**, 2444-2448.

Peberdy, J. F. (1985) Mycolytic enzymes. In: *Fungal Protoplasts: Applications in Biochemistry & Genetics*. (Eds. J.F. Peberdy & L. Ferenczy) pp.31-44. Marcel Dekker inc., New York.

Peng, J. T. (1986) Resistance to disease in *Agaricus bisporus*. PhD Thesis, The University of Leeds.

Perrakis, A., Tews, I., Dauter, Z., Oppenheim, A. B., Chet, I., Wilson, K. S. & Vorgias, C. E. (1994) Crystal-structure of a bacterial chitinase at 2.3 Angstrom resolution. *Structure* **2**, 1169-1180.

Pesole, G., Bozetti, M. P., Preparata, G. & Saccone, C. (1991) Glutamine synthetase gene evolution; a good molecular clock. *Proceedings of the National Academy of Sciences USA* **88**, 522-526.

Phillips, I. (1991) A guide to sensitivity testing. *Journal of Antimicrobial Chemotherapy* **27**, (Supplement D), 1-50.

Pien, F. D., Farmer III, J. J. & Weaver, R. E. (1983) Polymicrobial bacteraemia caused by *Ewingella americana* (Family *Enterobacteriaceae)* and an unusual *Pseudomonas* species. *Journal of Clinical Microbiology* **18**, 727-729.

Pitcher, D. G., Owen, R. J., Dyal, P. & Beck, A. (1987) Synthesis of a biotinylated DNA probe to detect ribosomal RNA cistrons in *Providencia stuartii*. *FEMS Microbiology Letters* **48**, 283-287.

Pitcher, D. G., Saunders, N. A. & Owen, R. J. (1989) Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Letters in Applied Microbiology* **8**, 151-156.

Platt, T. (1986) Transcription termination and the regulation of gene expression. *Annual Review of Biochemistry* **55**, 339-372.

Podila, G. K. & Flurkey, W. H. (1986) *In vitro* translation of mushroom tyrosinase. *Biochemical, Biophysical Research Communications* **141**, 697-703.

Poppe, J. (1978) Mushroom domestications, their disease-index and growth abnormalities. *Mushroom Science* **11**, 851-872.

Preece, T. F. & Wong, W. C. (1982) Quantitative and scanning electron microscope observations on the attachment of *Pseudomonas tolaasii* and other bacteria to the surface of *Agaricus bisporus*. *Physiological Plant Pathology* **21**, 251-257.

Prentki, P. & Krisch, H. M. (1984) *In vitro* insertional mutagenesis with a selectable DNA fragment. *Gene* **29**, 303-313.

Prévost, G., Pottecher, B., Dahlet, M., Bientz, M., Mantz, J. M. & Piémont, Y. (1991) Pulsed field gel electrophoresis as a new epidemiological tool for monitoring methicillin-resistent *Staphylococcus aureus* in an intensive care unit. *Journal of Hospital Infection* **17**, 255-269.

Priest, F. G., Kaji, D. A., Rosato, Y. B. & Canhos, V. P. (1994) Characterisation of *Bacillus thuringiensis* and related bacteria by ribosomal RNA gene restriction fragment length polymorphisms. *Microbiology* **140**, 1015-1022.

Prosser, J. I. (1994) Molecular marker systems for detection of genetically engineered micro-organisms in the environment. *Microbiology* **140**, 5-17.

Pugsley, A. P. & Schwartz, M. (1985) Export and secretion of proteins by bacteria. *FEMS Microbiology Reviews* **32**, 3-38.

Pugsley, A.P. (1993) The complete general secretory pathway in Gram-negative bacteria. *Microbiological Reviews* **57**, 50-108.

Pumpuni, C. B., Beier, M. S., Nataro, J. P., Guers, L. D. & Davis, J. R. (1993) *Plasmodium falciparum*: Inhibition of sporogonic development in *Anopheles stephensi* by Gram-negative bacteria. *Experimental Parasitology* **77**, 195-199.

Rabsch, W. & Winkelmann, G. (1991) The specificity of bacterial siderophore receptors probed by bioassays. *Biology of Metals* **4**, 244-250.

Raikhel, N. V. & Lee, H.-I. (1993) Structure and function of chitin binding proteins. *Annual Review of Plant Molecular Biology* **44**, 591-615.

Rainey, P. B. & Cole, A. L. J. (1988) A new bacterial disease of the cultivated mushroom, *Agaricus bisporus*. *Transactions of the British Mycological Society* **90**, 122-125.

Rainey, P. B. (1991a) Effect of *Pseudomonas putida* on hyphal growth of *Agaricus bisporus*. *Mycological Research* **95**, 699-704.

Rainey, P. B. (1991b) Phenotypic variation of *Pseudomonas putida* and *P. tolaasii* affects attachment to *Agaricus bisporus* mycelium. *Journal of General Microbiology* **137**, 2769-2779.

Rainey, P. B., Brodey, C. L. & Johnstone, K. (1991) Biological properties and spectrum of activity of tolaasin, a lipodepsipeptide toxin produced by the mushroom pathogen *Pseudomonas tolaasii*. *Physiological and Molecular Plant Pathology* **39**, 57-70.

Rainey, P. B., Brodey, C. L. & Johnstone, K. (1993) Identification of a gene cluster encoding 3 high molecular weight proteins, which is required for the synthesis of tolaasin by the mushroom pathogen *Pseudomonas tolaasii*. *Molecular Microbiology* **8**, 643-652.

Richardson, P. N. (1993) Stipe necrosis of cultivated mushrooms (*Agaricus bisporus*) associated with a fluorescent pseudomonad. *Plant Pathology* **42**, 927-929.

Robbins, P. W., Albright, C. & Benfield, B. (1988) Cloning and expression of a *Streptomyces plicatus* chitinase (chitinase-63) in *Escherichia coli*. *The Journal of Biological Chemistry* **263**, 443-447.

Robbins, P. W., Overbye, K., Albright, C., Benfield, B. & Pero, J. (1992) Cloning and high-level expression of chitinase-encoding gene of *Streptomyces plicatus*. *Gene* **111**, 69-76.

Roberts, R. L. & Cabib, E. (1982) *Serratia marcescens* chitinase: one step purification and use for the detection of chitin. *Analytical Biochemistry* **127**, 402-412.

Roberts, W. K. & Selitrennikoff, C. P. (1988) Plant and bacterial chitinases differ in antifungal activity. *Journal of General Microbiology* **134**, 169-176.

Roffey, P. E. & Pemberton, J. M. (1990) Cloning and expression of an *Aeromonas hydrophila* chitinase gene in *Escherichia coli*. *Current Microbiology* **21**, 329-337.

Romaguera, A., Menge, U., Breves, R. & Diekmann, H. (1992) Chitinases of *Streptomyces olivaceoviridis* and significance of processing for multiplicity. *Journal of Bacteriology* **174**, 3450-3454.

Royse, D. J. & Wuest, P. J. (1980) Mushroom brown blotch: effect of chlorinated water on disease intensity and bacterial populations in the casing soil and on the pilei. *Phytopathology* **70**, 902-905.

Rudall, K. M. & Kenchington, W. (1973) The chitin system. *Biological Reviews* **49**, 597-636.

Saborowski, W., Buchholz, F., Vetter, R. A. H., Wirth, S. J. & Wolf, G. A. (1993) A soluble, dye-labelled chitin derivative adapted for the assay of krill chitinase. *Comprehensive Biochemistry and Physiology* **105B**, 673-678.

Sahai, A. S. & Manocha, M. S. (1993) Chitinases of fungi and plants: their involvement in morphogenesis and host-parasite interaction. *FEMS Microbiology Reviews* **11**, 317-338.

Samadpour, M., Moseley, S. L. & Lory, S. (1988) Biotinylated DNA probes for Exotoxin A and Pilin genes in the differentiation of *Pseudomonas aeruginosa* Strains. *Journal of Clinical Microbiology* **26**, 2319-2323.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press. Cold Spring Harbor. 2nd Edition.

Samson, R., Houdeau, G., Khanna, P., Guillaumes, J. & Oliver, J. M. (1986). Variability of fluorescent *Pseudomonas* populations in composts and casing soils used for mushroom cultures. In: *Proceedings of the International Symposium on the Scientific and Technical Aspects of Cultivating Edible Fungi*, The Pennsylvania State University, PA, USA. pp.19-25.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences USA* **74**, 5463-5467.

Schisler, L. C., Sinden, J. W. & Sigel, E. M. (1968) Etiology of mummy disease of cultivated mushrooms. *Phytopathology* **58**, 944-948.

Schlumbaum, A., Mauch, F., Vogeli, U. & Boller, T. (1986) Plant chitinases are potent inhibitors of fungal growth. *Nature* **324**, 365-367.

Schmidt, T. M., Kopecky, K. & Nealson, K. H. (1989) Bioluminescence of the insect pathogen *Xenorhabdus luminescens*. *Applied and Environmental Microbiology* **55**, 2607-2612.

Schwyn, B. & Neilands, J. B. (1987) Universal chemical assay for the detection and determination of siderophores. *Analytical Biochemistry* **160**, 47-56.

Shaikh, S. A. & Deshpande, M. V. (1993) Chitinolytic enzymes: their contribution to basic and applied research. *World Journal of Microbiology and Biotechnology* **9**, 468-475.

Shapira, R., Ordentlich, A., Chet, I. & Oppenheim, A. B. (1989) Control of plant diseases by chitinase expressed from cloned DNA in *Escherichia coli*. *Phytopathology* **79**, 1246-1249.

Shaw, J. J., Dane, F., Geiger, D. & Kloepper, J. W. (1992) Use of bioluminescence for detection of genetically engineered microorganisms released into the environment. *Applied and Environmental Microbiology* **58**, 267-273.

Shimahara, K. & Takiguchi, Y. (1988) Preparation of crustacean chitin. *Methods in Enzymology* **161**, 417-423.

Sietsma, J. H. & Wessels, J. G. H. (1979) Evidence for covalent linkages between chitin and β-glucan in a fungal wall. *Journal of General Microbiology* **114**, 99-108.

Sitrit, Y., Barak, Z., Kapulnik, Y., Oppenheim, A. P. & Chet, I. (1993) Expression of a *Serratia marcescens* chitinase gene in *Rhizobium meliloti* during symbiosis on alfalfa roots. *Molecular Plant-Microbe Interactions* **6**, 293-298.

Sitrit, Y., Oppenheim, A. & Chet, I. (1994) Isolation and characterisation of a chitinase encoding gene from *Aeromonas caviae*. *Unpublished GenBank Submission*

Smith, J. F., Love, M. E. & Burton, K. S. (1993) Comparative studies of the quality of fresh and stored mushrooms of *Agaricus bisporus* with 2 tropical *Agaricus bitorquis* strains. *Annals of Applied Biology* **122**, 593-603.

Sonnhammer, E. L. L. & Durbin, R. (1994) A workbench for large scale sequence homology analysis. *Computing Applications in the Biosciences* **10**, 301-307.

Speert, D. P., Campbell, M. E., Farmer, S. W., Volpel, K., Joffe, A. M. & Paranchych, W. (1989) Use of a pilin gene probe to study molecular epidemiology of *Pseudomonas aeruginosa*. *Journal of Clinical Microbiology* **27**, 2589-2593.

Spruce, J., Mayer, A. M. & Osborne, D. J. (1987) A simple histochemical method for locating enzymes in plant tissue using nitrocellulose blotting. *Phytochemistry* **26**, 2901-2903.

Stanier, R. Y., Palleroni, N. J. & Doudoroff, M. (1966) The aerobic Pseudomonads: a taxonomic study. *Journal of General Microbiology* **43**, 159-271.

Stead, D. E. (1992a) Grouping of plant-pathogenic and some other *Pseudomonas* spp. by using cellular fatty-acid profiles. *International Journal of Systematic Bacteriology* **42**, 281-295.

Stead, D. E., Sellwood, J. E., Wilson, J. & Viney, I. (1992b) Evaluation of a commercial microbial identification system based on fatty acid profiles for rapid, accurate identification of plant pathogenic bacteria. *Journal of Applied Bacteriology* **72**, 315-321.

Stewart, G. S. A. B. & Williams, P. (1992) *lux* genes and the applications of bacterial bioluminescence. *Journal of General Microbiology* **138**, 1289-1300.

Stolzfus, A., Leslie, J. F. & Milkman, R. (1988) Molecular evolution of the *Eschericheria coli* chromosome. I. Analysis of structure and natural variation in a previously uncharacterised region between *trp* and *tonB*. *Genetics* **120**, 345-358.

Storey, I. F. (1954) Mummy disease of mushrooms. *Plant Pathology* **3**, 49.

Strivastava, A. K., Defago, G. & Boller, T. (1985) Secretion of a chitinase by *Aphanocladium album*, a hyperparasite of wheat rust. *Experientia* **41**, 1612-1613.

Tanaka, K., Takayanagi, Y., Fujita, N., Ishihama, A. & Takahashi, H. (1993) Heterogeneity of the principle σ factor of RNA polymerase in stationary-phase *Escherichia coli*. *Proceedings of the National Academy of Science, USA* **90**, 3511-3515.

Thapa, C. D. & Jandaik, C. L. (1989). Changes in polyphenol oxidase and perioxidase activity due to infection of *Verticillium fungicola* in fruit bodies of *Agaricus bisporus*. In: *Mushroom Science XII (Part II): Proceedings of the Twelfth International Congress on the Science and Cultivation of Edible Fungi*, Braunschweig - Germany, 1987 pp.765-769.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994) Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**, 4673-4680.

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

Tronsmo, A. & Harman, G. E. (1993) Detection and quantification of N-Acetyl-β-D-glucosaminidase, chitobiosidase, and endochitinase in solutions and on gels. *Analytical Biochemistry* **208**, 74-79.

Trudel, J. & Asselin, A. (1989) Detection of chitinase activity after polyacrylamide gel electrophoresis. *Analytical Biochemistry* **178**, 362-366.

Trujillo, R. (1968) Preparation of carboxymethylchitin. *Carbohydrate Research* **7**, 483-485.

Tsujibo, H., Endo, H., Minoura, K., Miyamoto, K. & Inamori, Y. (1993b) Cloning and sequence analysis of the gene encoding a thermostable chitinase from *Streptomyces thermoviolaceus*. *Gene* **134**, 113-117.

Tsujibo, H., Orikoshi, H., Tanno, H., Fujimoto, K., Miyamoto, K., Imada, C., Okami, Y. & Inamori, Y. (1993a) Cloning, sequence, and expression of a chitinase gene from a marine bacterium, *Alteromonas* sp. strain O-7. *Journal of Bacteriology* **175**, 176-181.

Tsuneda, A. & Thorn, G. (1994) Interactions between *Lentinula edodes* and pseudomonads. *Canadian Journal of Microbiology* **40**, 937-943.

Tucker, C. M. & Routien, J. B. (1942) The mummy disease of the cultivated mushroom. *University of Missouri College of Agriculture Research Bulletin* **358**, 27pp.

Turner, E. M. (1974) Phenoloxidase activity in relation to substrate and development stage in the mushroom, *Agaricus bisporus*. *Transactions of the British Mycological Society* **63**, 541-547.

Ueda, M., Kawaguchi, T. & Arai, M. (1994) Molecular cloning and nucleotide sequence of the gene encoding chitinase II from *Aeromonas* sp. No. 10S-24. *Journal of Fermentation and Bioengineering* **78**, 205-211.

Unsworth, B. A. & Preece, T. F. (1980) The taxonomy of *Pseudomonas* species associated with the commercial mushroom. *Journal of Applied Bacteriology* **49**, xiii-xiv.

Valadares, M. C. C. (1995) The Chitinolytic System in *Metarhizium anisopliae*. PhD Thesis, University of Nottingham.

Valadares, M. C. C., Inglis, P. W. & Peberdy, J. F. (1995) Cloning and partial sequence of a chitinase from the entomopathogenic fungus, *Metarhizium anisopliae*. *FEMS Microbiology Letters* (Submitted for publication).

Vamos-Vigyazo, L. (1981) Polyphenol oxidase and peroxidase in fruits and vegetables. *CRC Critical Reviews in Food Science and Nutrition* **15**, 49.

van Zaayen, A. & Wattereus, H. A. J. I. (1974) Intracellular occurrence of bacteria in mummy-diseased mushrooms. *Phytopathology* **64**, 1474-1475.

van Zaayen, A. (1981) Diseases and pests of cultivated mushrooms: bacterial diseases. *The Mushroom Journal* **102**, 193-196.

Vanscheltinga, A. C. T., Kalk, K. H., Beintema, J. J. & Dijkstra, B. W. (1994) Crystal-structure of hevamine, a plant defence protein with chitinase and lysozyme activity, and its complex with an inhibitor. *Structure* **2**, 1181-1189.

Vantomme, R., Overstijns, A., Goor, M., Kersters, K. & De ley, J. (1989). Routine diagnosis and sensitivity to chemical compounds of phytopathogenic and saprophytic pseudomonads from cultivated mushrooms. In: *Mushroom Science XII (Part II): Proceedings of the Twelfth International Congress on the Science and Cultivation of Edible Fungi*, Braunschweig - Germany, 1987 pp.701-710.

Verburg, J. G. & Huynh, Q. K. (1991) Purification and characterisation of an antifungal chitinase from *Arabidopsis thaliana*. *Plant Physiology* **95**, 450-455.

Verdonk, L., Mergaert, J., Rijckaert, C., Swings, J., Kersters, K. & De Ley, J. (1987) Genus *Erwinia*: numerical analysis of phenotypic features. *International Journal of Systematic Bacteriology* **37**, 4-18.

von Heijne, G. (1983) Pattern of amino acids near signal sequence cleavage sites. *European Journal of Biochemistry* **133**, 17-21.

Vyas, P. & Deshpande, M. V. (1993) Purification and characterisation of an endochitinase from *Myrothecium verrucaria*. *Journal of General and Applied Microbiology* **39**, 91-99.

Ward, D. M., Weller, R. & Bateson, M. M. (1990) 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature (London)* **345**, 63-65.

Watanabe, T., Suzuki, K., Oyanagi, W., Ohnishi, K. & Tanaka, H. (1990) Gene cloning of chitinase A1 from *Bacillus circulans* WL-12 reveals its evolutionary relationship to *Serratia* chitinase and to the type III homology unit of fibronectin. *Journal of Biological Chemistry* **265**, 15659-15665.

Watanabe, T., Oyanagi, W., Suzuki, K., Ohnishi, K. & Tanaka, H. (1992) Structure of the gene encoding chitinase D of *Bacillus circulans* WL-12 and possible homology of the enzyme to other prokaryotic chitinases and class III chitinases. *Journal of Bacteriology* **174**, 408-414.

Watanabe, T., Kobori, K., Yamada, T., Ito, Y., Uchida, M. & Tanaka, H. (1993a) Domain structures and functions of bacterial chitinases In: *Chitin Enzymology*. Edited by R.A.A. Muzzarelli. The European Chitin Society, Ancona, Italy. pp.329-336.

Watanabe, T., Kobori, K., Miyashita, K., Fujii, T., Sakai, H., Uchida, M. & Tanaka, H. (1993b) Identification of glutamic acid 204 and aspartic acid 200 in chitinase A1 of *Bacillus circulans* WL-12 as essential residues for chitinase activity. *Journal of Biological Chemistry* **268**, 18567-18572.

Watanabe, T., Ito, Y., Yamada, T., Hashimoto, M., Sekine, S. & Tanaka, H. (1994) The roles of the C-terminal domain and type III domains of chitinase A1 from *Bacillus circulans* WL-12 in chitin degredation. *Journal of Bacteriology* **176**, 4465-4472.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E., Stakebrandt, E., Starr, M. P. & Trüper, H. G. (1987) Report on the Ad Hoc committee on

reconciliation of approaches to bacterial systematics. *International Journal of Systematic Bacteriology* **37**, 463-464.

Weust, P. J. & Zarkower, P. A. (1991) Mummy disease of button mushrooms: Causation, crop loss, mycosphere implications In: *Science and Cultivation of Edible Fungi*. (Ed. M.J. Maher) pp.397-401. Balkema, Rotterdam.

Wilson, D. M. (1968) Physiology of sexual reproduction in *Hypomyces solani* f. sp. *cucurbitae*. V. Effect of tyrosinase. *Phytopathology* **58**, 1697-1699.

Wirth, S. J. & Wolf, G. A. (1990) Dye-labelled substrates for the assay and detection of chitinase and lysozyme activity. *Journal of Microbiological Methods* **12**, 197-205.

Woese, C. R. (1994) There must be a prokaryote somewhere: microbiology's search for itself. *Microbiological Reviews* **58**, 1-9.

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

Wong, W. C. & Preece, T. F. (1980) *Pseudomonas tolaasi* in mushroom crops: A note on primary and secondary sources of the bacterium on a commercial farm in England. *Journal of Applied Bacteriology* **49**, 305-314.

Wong, W. C. & Preece, T. F. (1982) *Pseudomonas tolaasii* in cultivated mushroom (*Agaricus bisporus*) crops: numbers of the bacterium and symptom development on mushrooms grown in various environments after artificial inoculation. *Journal of Applied Bacteriology* **53**, 87-96.

Wong, W. C., Fletcher, J. T., Unsworth, B. A. & Preece, T. F. (1982) A note on ginger blotch, a new bacterial disease of the cultivated mushroom, *Agaricus bisporus*. *Journal of Applied Bacteriology* **52**, 43-48.

Wong, W. C. & Preece, T. F. (1985a) *Pseudomonas tolaasii* in cultivated mushroom (*Agaricus bisporus*) crops: effects of sodium hypochlorite on the bacterium and on blotch disease severity. *Journal of Applied Bacteriology* **58**, 259-267.

Wong, W. C. & Preece, T. F. (1985b) *Pseudomonas tolaasii* in mushroom (*Agaricus bisporus*) crops: activity of formulations of 2 bromo-2-nitropropane-1,3-diol (Bronopol) against the bacterium and use of this compound to control blotch disease. *Journal of Applied Bacteriology* **58**, 275-282.

Wood, D. A. & Hammond, J. B. W. (1977) Inhibition of growth and development of *Agaricus bisporus* by Polyoxin D. *Journal of General Microbiology* **98**, 625-628.

Wood, D. A., Claydon, N., Burton, K. S., Matcham, S. E., Allan, M., Perry, C., Thurston, C. F., Raguz, S. & Yague, E. (1991) Molecular analysis of enzymes of *Agaricus bisporus*. In: *Science and Cultivation of Edible Fungi*. (Ed. M.J. Maher) pp.43-50. Balkema, Rotterdam.

Wortman, A. T., Somerville, C. C. & Colwell, R. R. (1986) Chitinase determinants of *Vibrio vulnificus*: Gene cloning and applications of a chitinase probe. *Applied and Environmental Microbiology* **52**, 142-145.

Yamaguchi, M., Hwang, P. M. & Campbell, J. D. (1970) Latent o-diphenol oxidase in mushrooms (*Agaricus bisporus*). *Canadian Journal of Biochemistry* **48**, 198-202.

Yao, R. J., Burr, D. H., Doig, P., Trust, T. J., Niu, H. Y. & Guerry, P. (1994) Isolation of motile and non-motile insertional mutants of *Campylobacter jejuni* - the role of motility in adherence and invasion of eukaryotic cells. *Molecular Microbiology* **14**, 883-893.

Yogev, D., Sela, S., Bercovier, H. & Razin, S. (1988b) Elongation factor (EF-Tu) gene probe detects polymorphism in *Mycoplasma* strains (FEM 03154). *FEMS Microbiology Letters* **50**, 145-149.

Young, J. M. (1970) Drippy gill: a bacterial disease of cultivated mushrooms caused by *Pseudomonas agarici*. *New Zealand Journal of Agricultural Research* **13**, 977-990.

Young, J. P. W. (1994) All those new names: an overview of the molecular phylogeny of plant-associated bacteria. In: *Advances in molecular genetics of plant-microbe interactions*. (Eds. M.J. Daniels) pp.73-80. Kluwer Academic Publishers, Amsterdam.

Zarkower, P. A., Wuest, P. J., Royse, D. J. & Myers, B. (1984) Phenotypic traits of fluorescent pseudomonads causing bacterial blotch of *Agaricus bisporus* mushrooms and other mushroom-derived fluorescent pseudomonads. *Canadian Journal of Microbiology* **30**, 360-367.

Zhu, Q. & Lamb, C. J. (1991) Isolation and characterisation of a rice gene encoding a basic chitinase. *Molecular and General Genetics* **226**, 289-296.