Project title:	Mushrooms: Identification, detection and control of different *Pseudomonas* species causing bacterial blotch symptoms
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Industry Representative:	
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The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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GROWER SUMMARY

Headline

- Causes of severe mushroom blotch in the UK have been previously identified as *Pseudomonas tolaasii* and '*Pseudomonas gingeri'*, causing severe brown blotch and ginger blotch respectively
- Characterisation of the bacterium by whole genome analysis has enabled development and confirmed the specificity of a molecular test for detection of '*P. gingeri*' and a previous test developed for *P. tolaasii*, the cause of severe brown blotch
- Irrigation of mushrooms with 0.3% w/w $CaCl_2$ solution reduced the incidence of blotched mushrooms under commercial conditions
- Incubation of mushroom casing samples in LB broth containing compound A may improve the detection of pathogenic *Pseudomonas* species

Background

This project aimed to provide tools to assist evaluation and development of potential control methods for bacterial blotch disease (caused by different *Pseudomonas* species). Bacterial blotch is considered to be the most important disease problem currently faced by the mushroom industry in the UK and elsewhere in Europe. Losses can exceed 30% of production. Since conditions that favour high yield are also favourable for disease development and transmission, there is a constant trade-off between maximising yield and maintaining health and quality of production. It is suspected that mushroom casing and compost may be sources of the bacteria. Current outbreaks of ginger blotch in N. Ireland are causing serious losses in mushroom production. The growers involved are anxious to understand the possible sources of infection, the production conditions most suitable for disease development and any potential methods for disease control. Practical and sustainable measures that can provide early warning of the presence of botch-causing bacteria, or can effectively reduce or eliminate the impact of their presence, are expected to have benefits across all stages of the supply chain resulting in reduced losses, reduced production costs and guaranteed sustainable supply of high quality end-product.

Summary

1. Severe mushroom blotch in the UK was previously confirmed to be caused by *Pseudomonas tolaasii* and '*Pseudomonas gingeri'*, causing severe brown blotch and ginger blotch respectively.
2. Phylotypes of other *Pseudomonas* strains that can cause mild blotch symptoms, or that are not pathogenic to mushroom, were differentiated at the species level from *P. tolaasii* and '*P. gingeri*' by whole genome analyses.
3. Surveys of commercial mushroom production in the Netherlands and Belgium have found the same species of bacteria causing severe blotch as in the UK, although other, as yet unnamed, species are also suspected to be able to cause severe blotch.
4. Molecular tests using quantitative real time (TaqMan) polymerase chain reaction were identified that detected either the *P. tolaasii* or '*P. gingeri*' strains that caused severe blotch in the UK, but did not cross react with other non-pathogenic *Pseudomonas* strains or strains that cause mild blotch symptoms.
5. Genome sequence of other blotch-causing *Pseudomonas* phylotypes is available for potential further diagnostic development if they are found to be commercially important.
6. Incubation of casing samples in LB broth containing compound A or compound B resulted in a greater pseudomonad population than incubation of casing samples in LB broth alone.
7. Incubation of mushroom casing samples in LB broth containing compound A resulted in a selectively greater increase in the *Pseudomonas* population when *P. tolaasii* or '*P. gingeri*' were present in the samples.
8. Preliminary results suggested that enrichment of inoculated casing samples in LB broth containing compound A could improve the detection of *P. tolaasii* or '*P. gingeri*' using the newly developed molecular tests.
9. Irrigation of mushrooms with a 0.3% $CaCl_2$ solution resulted in significantly fewer blotched mushrooms than irrigating with the same volume of water, both at high and low levels of relative humidty.
10. There was no difference in the level of blotched mushrooms between several other casing treatments tested.
11. There were no significant effects of the growing room humidity, of 0.3% $CaCl_2$ and other casing treatments used on the yield of mushrooms (range of treatment averages 321-367 g/kg compost).
12. The populations of total cultural bacteria and of *Pseudomonas* in the casing was not affected by irrigation with a 0.3% CaCl2 solution nor by the other casing treatments used.

Financial Benefits

- This project should lead to improved diagnostic tests for pathogenic pseudomonads resulting in the identification of casing materials that are less prone to bacterial blotch
- If $CaCl_2$ solution can be registered for control of bacterial blotch, this could significantly reduce the incidence of the disease

Action Points

- Further work is needed, possibly with other EU countries, in registering $CaCl_2$ solution as a basic material for use on mushrooms

SCIENCE SECTION

Introduction

In HDC Project M54 (2011-12), the development of bacterial blotch symptoms under different environmental conditions was studied and a real-time PCR test for *Pseudomonas tolaasii* based on the tolaasin toxin gene sequence was developed. This test successfully detected the bacterium in both casing materials and symptomatic mushrooms following inoculation of the casing with *P. tolaasii*. However, *Pseudomonas* isolates from mushrooms with either severe ginger blotch or mild blotch symptoms, and which developed on uninoculated casing from specific sources, were not detected with this test. All of these isolates, together with a collection of isolates from diseased mushrooms obtained during previous research at Horticulture Research International (HRI), were preserved at Fera within the National Collection of Plant Pathogenic Bacteria (NCPPB) through HDC Project M59 (2012). There remains confusion over the phylogenetic relationships between the various *Pseudomonas* pathogens causing bacterial blotch, including those currently named as *P. tolaasii*, *P. gingeri* and other unknown *Pseudomonas* isolates that cause mild blotch under conducive conditions. Van der Wolf *et al.* (2016) used a multilocus sequence analysis to characterise different species of *Pseudomonas* that cause brown blotch; they based their characterisation of pseudomonads on pathogenicity tests on detached mushroom caps. A similar technique for screening bacteria causing blotch symptoms is based on using inoculated cubes of mushroom cap tissue (Gandy, 1968; Godfrey *et al.*, 2001). In project M54, *P. tolaasii* strains that caused typical blotch symptoms also produced dark brown pigmentation in the cap tissue bioassay; a *P. gingeri* isolate that produced typical ginger blotch in culture produced a pale brown discolouration in the cap tissue bioassay. Non-pathogenic pseudomonads did not cause cap tissue discolouration but it is not clear if the detached cap or tissue bioassays can distinguish mild blotch causing pseudomonads from non-pathogenic pseudomonads or *P. gingeri*. The influence of the casing environment and microbiota on the blotch pathogenicity of *Pseudomonas* sp. isolates is also not considered in the cap tissue bioassay.

In HDC Project M60 (2015) all the fresh samples of casing examined tested negative using the Taqman PCR *Pseudomonas tolaasii* test, irrespective of subsequent blotch disease incidence. Casing samples taken after the second flush gave positive test results, partially corresponding with the occurrence of blotch disease. This indicates that (1) the level of *P. tolaasii* in the fresh casings was below the detectable limit (2) casing or environmental conditions favouring the development of *P. tolaasii* were more important than the initial population and/or (3) the source of the *P. tolaasii* was the Phase III compost and not the casing.

There is some evidence in the USA (Spear *et al.*, 1994) that Phase III compost used for caccing may be a source of *P. tolaasii* and that the use of sterile casing inoculum reduces the risk of blotch. The optimum pH for Pseudomonads is 7 compared with a normal casing pH of 7.5. The use of hydrated lime in casing increases the pH temporarily above 11; it is not clear whether this alkaline treatment would reduce the population of *P. tolaasii*.

The risk of blotch or mild discolouration has meant that watering on mushroom farms is usually avoided between airing and the end of the first flush. The use of 0.3% calcium chloride in irrigation water has been shown to reduce bacterial populations and the incidence of blotch (Munsch *et al.*, 2002). If the blotch risk can be avoided by the introduction of effective control measures, pre-first flush watering could produce higher mushroom yields and quality. An experiment conducted in HDC project M62 has shown that watering the casing with dilute glucose solution can significantly increase the population of pseudomonads in the casing without causing blotch. This may therefore be a potential method for increasing a casing population of bacteria that is antagonistic to *P. tolaasii*.

Current outbreaks of ginger blotch in N. Ireland are causing serious losses in mushroom production. The growers involved are anxious to understand the possible sources of infection, the production conditions most suitable for disease development and any potential methods for disease control. In particular, suggestions that infections are associated with particular sources of casing materials require urgent investigation.

Project aims:

1. To develop a small-scale blotch pathogenicity test for *Pseudomonas* isolates and compare pathogenicity determined from cap tissue bioassay.
2. To generate and compare whole genome sequence data from pathogenic *Pseudomonas* isolates and select suitable sequence diversity for development of improved diagnostic methods which can detect species causing typical bacterial blotch, ginger blotch or mild blotch symptoms.
3. To develop practical control measures for the different types of bacterial blotch by manipulating the casing bacterial population by cultural methods.

Project objectives:

1. Review the phylogenetic and ecological relationships between the various fluorescent *Pseudomonas* species causing bacterial blotch disease and those which are beneficial for mushroom production and may contribute to disease suppression.
2. Compare blotch pathogenicity obtained from small-scale culture test and cap tissue bioassay.

3. Generate whole genome sequences of *P. tolaasii*, *P. gingeri* and mild-blotch-causing *Pseudomonas* sp. isolates using Illumina MiSeq next generation sequencing, and perform bioinformatic analysis to compare whole genomes.
4. Select DNA sequences that (i) are unique to pathogenic isolates and not found in non-pathogenic *Pseudomonas* species (ii) distinguish isolates causing typical bacterial blotch, ginger blotch or mild blotch symptoms.
5. Use selected sequences to develop improved diagnostic tests (e.g. real-time PCR or LAMP assays) which detect and differentiate *Pseudomonas* species pathogenic to mushrooms in both casing and mushroom samples, and evaluate performance of new tests using reference isolates, casing and Phase III compost samples and symptomatic mushrooms from commercial sources.
6. Investigate control of different types of bacterial blotch by manipulation of the casing bacterial population by the use of casing additives and casing inoculum, and calcium chloride addition to the irrigation water.

1. Materials and methods

1.1. Review of fluorescent *Pseudomonas* species causing bacterial blotch disease.

A short literature review was conducted with the aims to:

- Clarify the phylogenetic and ecological relationships between pathogenic and beneficial fluorescent *Pseudomonas* species commonly found in mushroom casing/compost
- Assess methods that have been previously used to control bacterial blotch and identify those with potential for further development

1.2. Comparison of blotch pathogenicity in small-scale pot tests and *in vitro* cap bioassays.

Two methods, a culture test in inoculated pots and a cut cap tissue bioassay, were compared to confirm blotch pathogenicity amongst a panel of *Pseudomonas* species isolated from mushroom. A panel of 27 *Pseudomonas* sp. isolates, including isolates from recent severe ginger blotch disease outbreaks during commercial production in the UK, were used in the pathogenicity tests (Table 1). These included isolates assigned to *P. tolaasii* and '*P. gingeri*' and known to be pathogenic from previous cap tissue and/or pot culture bioassays, non-pathogenic *Pseudomonas* isolates, an isolate of *Pseudomonas agarici* which causes drippy gill symptoms, and various other isolates with unknown pathogenicity. Reference strains of other Pseudomonas species, strains of which have been reported to cause mushroom blotch (including *P. costantinii*, '*P. reactans*' and *P. protegens*) were also included.

Table 1. Fluorescent *Pseudomonas* isolates used in the pathology studies

#	Collection identification number			Country of origin	Year isolated	Identity originally assigned
	Protect	**NCPPB**	**Other**			
1		3146	LMG 2343	UK	1981	*'Pseudomonas gingeri'*
2	P7548	3637	CFBP 4832	UK	1989	*'Pseudomonas gingeri'*
3	P7758		ATCC 51311; CFBP 5116	USA	?	*'Pseudomonas gingeri'*
4	P7779		ATCC 51312; CFBP 5114	USA	?	*'Pseudomonas gingeri'*
5	P8018			UK	2011	*'Pseudomonas gingeri'*
6			21614711	UK	2016	*'Pseudomonas gingeri'*
7			21615525	UK	2016	*'Pseudomonas gingeri'*
8			21615526	UK	2016	*'Pseudomonas gingeri'*
9	P7544	2192^T		UK	1965	*Pseudomonas tolaasii*
10	P7756		ATCC 51309	USA	?	*Pseudomonas tolaasii*
11	P7757		ATCC 51310	USA	?	*Pseudomonas tolaasii*
12		3149	LMG 5329	UK	1979	'*Pseudomonas reactans*'
13	P7759		ATCC 14340	UK	1957	*'Pseudomonas reactans'*
14		1311		UK	1962	*'Pseudomonas reactans'*
15		4616	CFBP 5705^T; LMG 22119	Finland	1997	*Pseudomonas costantinii*
16		2289^T		New Zealand	1970	*Pseudomonas agarici*
17	P7760		HRI CH6			*Pseudomonas agarici*?
18	P7772		HRI mar-12			*Pseudomonas veronii*
19	P7774		HRI n12			*Pseudomonas poae*?
20		4617	CFBP 6595^T	Switzerland	1986	*Pseudomonas protegens*
21	P7771		HRI WB1			*Pseudomonas putida*?
22	P7765		HRI S. Lincoln T2/6	UK	1980s	*Pseudomonas putida*?
23	P7753		HRI -			*Pseudomonas syringae*?
24	P7786			UK	2011	*Pseudomonas* sp.
25	P7787			UK	2011	*Pseudomonas* sp.
26	P8021			UK	2011	*Pseudomonas* sp.
27		2193		UK	1968	*Pseudomonas tolaasii*

NCPPB = National Collection of Plant Pathogenic Bacteria, Sand Hutton, York, UK.
CFBP = French Collection of Plant Pathogenic Bacteria, INRA, Angers, France.
ATCC = American Type Culture Collection, Rockville, Maryland, USA

(a) Pot test

To assess the effect of the various isolates of *Pseudomonas* species on disease incidence, mushrooms were grown in plastic pots, 230 mm diameter x 220 mm depth, each containing 4 kg of Phase III compost, spawn-run with the mushroom strain Sylvan A15. The pots were cased with 1.3 L of casing material (moist mixture of peat and sugar beet lime). The casing did not contain compost caccing to reduce potential contamination with additional pathogenic pseudomonads. The pots were watered with sterile distilled water (STW) and kept in a

growing room at 25 °C for 7 days; the room was then 'aired' and the air temperature reduced to 18 °C and the relative humidity was maintained at 91-93%. After a further 5 days the pots were inoculated by evenly watering the casing in replicate pots (two for each isolate) with 50 ml of an aqueous suspension of each bacterial isolate containing approximately 10^7 colony-forming units (cfu) per mL. Four negative controls were watered with sterile distilled water only. Disease development on the mushrooms was recorded daily over two flushes as severe or mild ginger or brown blotch, or no disease.

(b) Cut cap tissue bioassay

A cut-cap bioassay was based on the method of Godfrey *et al*. (2001). In brief, the stipes of white button mushrooms were removed and the caps were placed in large Petri dishes on damp filter paper. Drops (10 µL) of bacterial inoculum (aqueous suspensions containing 10^7 cfu per mL were placed onto each of 3 caps per *Pseudomonas* isolate and the dishes incubated at 21 ºC for 72 hours. Drops of sterile distilled water were used for controls. Symptom development was visually assessed daily and photographed up to 72 hrs.

1.3. Generation of whole genome sequences of recent blotch-causing isolates of *P. tolaasii*, *P. gingeri* and other non-pathogenic *Pseudomonas* sp. and bioinformatic analysis to compare these with other sequenced isolates.

Whole genome sequencing was performed using the Illumina Miseq next generation sequencing platform. Genome assembies were aligned and analysed using the calculate_ani.py script of the pyani software program (v0.2.7). Sequence was obtained for 18 *Pseudomonas* isolates (including 16 from the UK and two from the USA) for which sequence was not already available in public databases. Additional genome sequences were obtained from public databases at http://www.pseudomonas.com, https://www.ncbi.nlm.nih.gov/nuccore and https://www.ncbi.nlm.nih.gov/bioproject. Newly generated genome sequences were also shared through collaboration with J. van der Wolf and T. Taparia at Wageningen Plant Research (WUR) in the Netherlands, thus allowing access to additional genome sequences from blotch-causing isolates obtained in the Netherlands and Belgium. Genome sequences obtained from all of the *Pseudomonas* isolates were then aligned and compared using average nucleotide identity (ANI) analysis to determine the phylogenetic similarity between each isolate (Pritchard *et al*. 2016).

1.4. Selection of DNA sequences that (i) are unique to pathogenic isolates and not found in non-pathogenic *Pseudomonas* species (ii) distinguish isolates causing typical bacterial blotch, ginger blotch or mild blotch symptoms.

Primer and probe sequences were designed in a previous project (HDC Project M054, 2012) for specific detection of *P. tolaasii* using real-time TaqMan PCR. The specificity of this assay was reassessed by screening the sequenced genomes for presence of the primer and probe sequences. Similarly, the specificity of new TaqMan primer and probe sequences with specificity to '*P. gingeri*' isolates, designed at Wageningen Plant Research (WUR), provided by J. van der Wolf, was assessed by screening the sequenced genomes for the primer/probe sequences.

In addition, both TaqMan assays were performed on the panel of pathogenic and non-pathogenic mushroom isolates listed in Table 1. DNA was crudely extracted by heating aqueous suspensions containing approximately 10^6 cfu per mL of each strain to 96 °C for 4 min. Real-time PCR was performed using Applied Biosystems Prism 9700HT instrument, and data were analyzed with sequence detection system V. In all cases, 1 µl of DNA extract was used in 24 µl of master mix, and all samples were tested in duplicate. Negative controls containing nuclease-free water in the place of DNA were included in all runs. Real-time PCR was conducted using Taq-Man core reagents (Applied Biosystems) consisting of 1 x buffer A (50 mM KCl, 10 mM Tris-HCl, pH 8.3, carboxy-X-rhodamine passive reference dye), 5.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, and 0.625 U AmpliTaq Gold. All primers were used at a final concentration of 300 nM and all probes at a final concentration of 100 nM. Universal cycling conditions were 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles each consisting of 15 s at 95 °C and 1 min at 60 °C. Results were analyzed in terms of the average cycle threshold (C_T) values (cycle at which a positive PCR signal is first detected as determined by the algorithm built into the software).

1.5. Develop improved diagnostic tests which detect and differentiate pathogenic *Pseudomonas* species in casing, Phase III compost and mushroom samples.

1.5.1. Effect of compounds on *Pseudomonas tolaasii* suspensions

Cultures of two *Pseudomonas tolaasii* isolates obtained from the HRI culture collection, TRF 49 and TRF 59 (obtained by Terry Fermor from blotch diseased mushrooms in March 1986), were prepared on LB medium. These were used to prepare stock suspensions in LB broth. Flasks (250 ml) containing 50 ml of LB broth were then inoculated with stock solution of the appropriate isolate and different volumes of compound A.

The flasks were incubated at 20 °C on a shaker for 16 h. The suspensions from each flask were then plated in a dilution series on plates of LB agar and *Pseudomonas* isolation agar (PIA) media to determine the number of colony forming units (CFU) per ml of broth. There were two replicate flasks of each concentration of volatile, and two replicate plate determinations from each flask on each type of agar medium.

1.5.2. Effect of compounds on *Pseudomonas* species in casing samples.

The pre-enrichment of *Pseudomonas tolaasii* in LB broth suspension in a dilution series of compound A and compound B showed that two isolates could tolerate specified concentrations in pure culture. Suspensions of casing from the pathogenicity testing pot experiment were then tested using this concentration of these two compounds. Casing inoculated with *P. gingeri*, *P. tolaasii* or *P. costantinii* and uninoculated casing samples from the pathogenicity experiment (Table 2) were used for the tests. Casing (5 g) was placed in LB broth (45 ml) in 250 ml flasks. Samples were tested after 16 h for the total cultivable bacterial and pseudomonad populations using two replicate plates of LB and PIA media respectively.

Samples of casing material from the pot pathogenicity experiment, taken following the second flush, were also tested using the TaqMan qPCR assays for detection of *P. tolaasii* and '*P. gingeri*', before and after the enrichment treatment with compound A in LB broth suspension. The suspensions were centrifuged at 10,000g for 10 min at 4 °C to form a pellet and DNA was extracted from the pellet using the PowerMax Soil DNA isolation kit as per the manufacturer's instructions (MoBio, USA). The resulting DNA was then PCR amplified in duplicate as described above (Section 1.4).

Table 2. Isolates used in pathogenicity pot experiment and in enrichment tests.

#	Protect	NCPPB	Other	Identity
5	P8018			*Pseudomonas gingeri*
6			21614711	*Pseudomonas gingeri*
7			21615525	*Pseudomonas gingeri*
9	P7544	2192^{T}		*Pseudomonas tolaasii*
10	P7756		ATCC 51309	*Pseudomonas tolaasii*
11	P7757		ATCC 51310	*Pseudomonas tolaasii*
15		4616	CFBP 5705^{T} = LMG 22119	*Pseudomonas costantinii*
24	P7786			*Pseudomonas gingeri*
28	Inoculated with water only			
29	Inoculated with water only			
30	Inoculated with water only			
31	Inoculated with water only			

1.6. Investigate control of different types of bacterial blotch by manipulation of the casing bacterial population.

Experiments were conducted to assess the effects of different casing and cultural treatments on mushroom yield and bacterial disease incidence. Mushrooms were grown in plastic pots, 230 mm diameter x 220 mm depth, each containing 4 kg of Phase III compost, spawn-run with the mushroom strain Sylvan A15. The pots were cased with 1.3 L of casing materials (moist mixtures of peat and sugar beet lime or hydrated lime). Caccing (spawn-run compost of the strain A15) was added to casing at 6.5 kg/m^3 unless casing inoculum (CI) was used (at 2 kg/m^3). The pots were watered and kept in a growing room at 25 °C for 6 days; the room was then 'aired' and the air temperature reduced to 18 °C. The relative humidity was kept at 85-87% (Experiment 1) or 91-93% (Experiment 2). Two flushes of mushrooms were picked from the pots.

The numbers of healthy and blotch diseased mushrooms, and yield of healthy mushrooms were recorded. In each experiment, the following casing and cultural treatments were examined:

(a) Irish peat + sugar beet lime (Harte casing)
(b) German peat + sugar beet lime (Sterckx casing)
(c) Irish peat + hydrated lime
(d) Sterile casing inoculum (CI) (Harte casing)
(e) Glucose solution, 2% w/v (Harte casing)
(f) Calcium chloride solution, 0.3% w/v (Harte casing)
(g) Ultimate Mushroom Boost (JF McKenna), 20 ml/m^2 (Harte casing).

Treatments (e) and (f) were applied in the irrigation water in each watering. Treatment (g) was applied as a single application before the first and second flushes. It contained suspensions of *Pseudomonas putida* and *Bacillus subtilis* (both at 10^6 CFU/ml).

Determination of bacterial populations in casing

The casing population of *Pseudomonas* and total cultivable bacterial species at the start and end of the experiments were determined by plating casing extracts. *Pseudomonas* sp. were determined by using *Pseudomonas* isolation agar (PIA). Total cultivable bacterial species were determined on LB agar medium.

2. Results

2.1. Review of fluorescent *Pseudomonas* species causing bacterial blotch disease.

The literature review with 94 references is attached in full as Appendix 1 to this report. The main conclusions of the review were:

2.1.1. Bacterial blotch is caused by a diverse group of bacteria in the genus *Pseudomonas*. Variation in symptom severity/incidence and inconsistencies and overlaps in the names assigned to blotch-causing bacteria have frequently led to confusion as to the causative agents of the various types of blotch.

2.1.2. Severe brown blotch is mostly caused by bacteria in the *Pseudomonas fluorescens* species complex, which produce toxic peptides known as tolaasins that contribute to symptom development. These include *Pseudomonas tolaasii*, mostly known as the causative agent of severe brown blotch. Other tolaasin-producing strains in the *P. fluorescens* species complex that can also cause severe blotch symptoms, have been assigned to the species *P. costantinii* and *P. fluorescens*.

2.1.3. Tolaasins have also been found to be produced by strains of bacteria assigned to the *P. syringae* species complex and *P. aeruginosa*, although the pathogenicity of these strains requires confirmation. *P. aeruginosa* has been found on mushrooms and mushroom casing.

2.1.4. Another group of strains in the *Pseudomonas fluorescens* species complex have been invalidly named as '*Pseudomonas gingeri*', on the basis that they cause ginger blotch symptoms. Outbreaks of severe ginger blotch have recently been observed in Europe. Diagnostic tests based on detection of specific tolaasin gene sequences do not appear to detect '*P. gingeri*' strains.

2.1.5. Mild blotch symptoms can also be caused by bacteria that are not known to produce tolaasin toxins. Such strains have been assigned to *P. protegens*, the invalidly-named species '*P. reactans'* and *P. agarici* (better known as the cause of drippy gill disease of mushrooms).

2.1.6. Phylogenetic analysis in the USA and the Netherlands has shown significant diversity amongst *Pseudomonas* isolates from mushrooms with blotch symptoms. The degree to which this diversity relates to the current nomenclature within known blotch-causing species requires further clarification. Further investigation is also needed to confirm the pathogenicity of the different phylotypes identified and to relate these to current outbreaks of mushroom blotch so that appropriate detection tests and control strategies can be developed.

2.1.7. Non-pathogenic *Pseudomonas* species present in mushroom casing are important because they stimulate primordium formation. The most significant of these have been assigned to *P. putida,* although other *Pseudomonas* species may also be involved. However, these strains are genetically diverse, and many strains originally classified as *P. putida* biovar B (including the type strain of *P. putida*) have been reclassified into the *P. fluorescens* species complex. Further investigation is required to be able to correctly identify these beneficial bacteria and to differentiate them from the pathogenic *Pseudomonas* species.

2.1.8. Some non-pathogenic *Pseudomonas fluorescens* strains have been shown to have biocontrol activity against the blotch-causing bacteria. A strain of *Bdellovibrio bacteriovorus* also protected mushrooms from brown blotch caused by *P. tolaasii*. However, no commercial biocontrol products are currently available.

2.1.9. Current blotch controls rely on controlling humidity (<90%) and air flow to avoid condensation of water on the mushroom cap in which the pathogenic bacteria can multiply, whilst avoiding scaling that occurs when the humidity is too low. However, there are some contradictions in the relative humidities and air flows which have been shown to cause blotch and scaling. Avoiding fluctuations in temperature and humidity, and the resulting condensation on mushrooms, may be as or more important than maintaining specific constant values.

2.1.10. Improvements to the sensitivity and specificity of detection methods are needed so that casing and caccing materials can be reliably tested for the presence of low populations of the full range of pathogenic *Pseudomonas* species and thus eliminate them as potential primary sources of infection.

2.1.11. Efficacy of a number of disinfectants against *P. tolaasii* has been proven. These include 70% isopropanol, 10% sodium hypochlorite bleach, organic potassium salts (Premisan), hydrogen peroxide (OxiDate), dodecyl dimethyl ammonium chloride (Verticide) and penolic compounds (Environ).

2.1.12. Experimental chlorination of irrigation water using chlorine, sodium hypochlorite or citric acid-activated has reduced bacterial blotch by various degrees but none of these are registered for use on mushrooms in the UK. Other water treatments with potential to reduce blotch include 70-80 mM acetic acid, 0.3% calcium chloride, 0.75% hydrogen peroxide and ozonation.

2.2. Comparison of blotch pathogenicity in small-scale pot tests and *in vitro* cap bioassays.

The results of pathogenicity testing identified a small number of UK *Pseudomonas* isolates that were able to induce severe brown or ginger blotch symptoms (Table 3 and 5). Results from the *in vitro* cut cap tests in the laboratory were in good agreement with results from the

pot tests conducted in the controlled environment room. In all cases, pathogenic strains produced symptoms in both replicated pots and on at least three replicated cut mushroom caps. Three isolates previously assigned to *P. tolaasii* (NCPPB 2192^T, ATCC 51309 and ATCC 51310) were confirmed as causes of severe brown blotch. Three isolates obtained from recent outbreaks of ginger blotch (P8018, 21614711 and 21615525) were confirmed as causes of severe ginger blotch. Other isolates previously assigned to '*P. gingeri*' (NCPPB 3146, NCPPB 3637, ATCC 51311, ATCC 51312) or obtained from symptomatic mushrooms from the same recent ginger blotch outbreaks (21615526) did not induce blotch symptoms.

A reference isolate of *P. costantinii* (CFBP 5705^T) was also confirmed as a cause of brown blotch, although with less severe symtoms (Table 4 and 5). Other isolates (P7787 and P8021), suspected in a previous project (HDC M54) to cause mild blotch symptoms, failed to induce any symptoms, with the exception of P7786, which induced mild ginger blotch symptoms in both replications of the pot test (Table 4) and caused mild symptoms in the cut cap test (Table 5). All of the other *Pseudomonas* strains isolated from mushroom, failed to induce blotch symptoms in either test. These included isolates assigned to '*P. reactans*' (NCPPB 1311, NCPPB 3149 and ATCC 14340), *P. agarici* (NCPPB 2289^T and HRI CH6), *P. protegens* (CFBP 6595^T) and *P. syringae* (P7753), strains of which species have been previously reported to be able to cause blotch. Strains assigned to *P. putida* (P7771 and P7765), *P. veronii* (P7772) and *P. poae* (P7774), usually considered as beneficial pseudomonads, were also confirmed to be non-pathogenic.

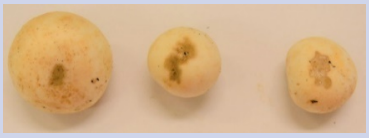
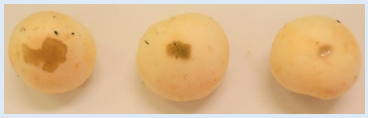
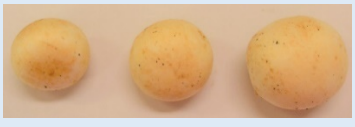
Table 3. *Pseudomonas tolaasii* and '*P. gingeri*' isolates causing severe brown blotch and severe ginger blotch respectively, in pot tests.

#	Original ID	Strain (origin)	Symptoms in pot test	% mushrooms with symptoms	
				Flush 1	Flush 2
9	*P. tolaasii*	P7544 = NCPPB 2192^{T} (UK, 1965)		96.3 (+/-1.1)	37.8 (+/-1.6)
10	*P. tolaasii*	P7756 = ATCC 51309 (USA)		100	82.7 (+/-3.5)
11	*P. tolaasii*	P7757 = ATCC 51310 (USA)		100	87.1 (+/-12.4)
5	'*P. gingeri*'	P8018 (UK, 2011)		99.3 (+/- 0.7)	50.0 (+/-1.0)
6	'*P. gingeri*'	21614711 (UK, 2016)		100	74.8 (+/-3.9)
7	'*P. gingeri*'	21615525 (UK, 2016)		100	62.8 (+/-1.6)

Table 4. *Pseudomonas* isolates causing mild or no blotch symptoms in pot tests.

#	Original ID	Strain (origin)	Symptoms in pot test	% mushrooms with symptoms	
				Flush 1	Flush 2
15	*P. costantinii*	CFBP 5705^T = LMG 22119^T = NCPPB 4616^T (Finland, 1997)		66.1 (+/-12.3)	19.0 (+/-5.1)
24	'*P. gingeri*'	P7786 (UK, 2011)		100	32.6 (+/-24.5)
1	'*P. gingeri*'	NCPPB 3146 (UK, 1981)		0	0
3	'*P. gingeri*'	P7758 = ATCC 51311 = CFBP 5116 (USA)		0	0
4	'*P. gingeri*'	P7779 = ATCC 51312 = CFBP 5114 (USA)		0	0
28	Control (H_2O)			0	0
29	Control (H_2O)			0	0

Table 5. Results from *in vitro* cut cap tests showing development of brown blotch following inoculation with pathogenic *Pseudomonas tolaasii* or *P. costantinii* isolates, ginger blotch following inoculation with three pathogenic '*P. gingeri*' isolates and no symptom development following inoculation with non-pathogenic isolates of '*P. gingeri*' (NCPPB 3146 and P7448) and sterile distilled water control.

#	Original ID	Strain (origin)	Symptoms (pot test)
9	*P. tolaasii*	P7544 = NCPPB 2192^{T} (UK, 1965)	
10	*P. tolaasii*	P7756 = ATCC 51309 (USA)	
11	*P. tolaasii*	P7757 = ATCC 51310 (USA)	
15	*P. costantinii*	CFBP 5705^{T} = LMG 22119^{T} = NCPPB 4616^{T} (Finland, 1997)	
5	'*P. gingeri*'	P8018 (UK, 2011)	
6	'*P. gingeri*'	21614711 (UK, 2016)	
7	'*P. gingeri*'	21615525 (UK, 2016)	
24	'*P. gingeri*'	P7786 (UK, 2011)	
1	'*P. gingeri*'	NCPPB 3146 (UK, 1981)	
3	'*P. gingeri*'	P7758 = ATCC 51311 = CFBP 5116 (USA)	
28	Control (H_2O)		

2.3. Generation of whole genome sequences of recent blotch-causing isolates of *P. tolaasii*, '*P. gingeri*' and other non-pathogenic *Pseudomonas* sp. and bioinformatic analysis to compare these with other sequenced isolates.

Whole genome sequences were obtained for 18 isolates studied (listed in Fig. 1). Sequences were not obtained for the severe brown blotch-causing isolate ATCC 51309 or the severe ginger blotch-causing isolate 21615525, which became mixed during preparation. Additional genome data, from 47 isolates from mushrooms with bacterial blotch collected at locations in the Netherlands and Belgium in 2015, was also provided for genome comparisons by WUR. Average nucleotide identity (ANI) analysis (Fig. 1) recognised 15 clusters of phylogenetically distinct genome sequences, differing at species level (<95% similar, Gorris *et al*. 2007). According to genome similarity, isolates causing severe brown blotch clustered together (phylotype 7) with the type strain of *P. tolaasii* (NCPPB 2192). Similarly, recent UK isolates which caused severe ginger blotch (P8018 and 21614711), clustered together in phylotype 8 with the non-pathogenic reference strain of '*P. gingeri*' (NCPPB 3146). The mild brown blotch-causing *P. costantinii* strain (NCPPB 4616) clustered with another reference strain of the same species (phylotype 5) but was phylogenetically distinct from the other blotch causing isolates. The mild ginger blotch-causing isolate (P7786) clustered in a phylogenetically distinct group of isolates (phylotype 9), which also included other blotch-causing isolates from the Netherlands/Belgium. Another cluster of related strains (phylotype 15) comprised a non-pathogenic recent UK isolate (21615526) and two non-pathogenic reference strains, which have been previously assigned to both *P. tolaasii* (as ATCC 51311 and ATCC 51312) and '*P. gingeri*' (as CFBP 5116 and CFBP 5114 respectively) according to Wells *et al*. (1996), but did not cluster with reference strains of either species. Several blotch-causing isolates from the Netherlands/Belgium survey also clustered in phylotype 15. Genome comparisons for some of the non-pathogenic fluorescent *Pseudomonas* isolates, resulted in some clustering with reference isolates of '*P. reactans'* (phylotype 6) or *P. veronii* (phylotype 4). Other phylotypes (2, 3 and 10-14) were represented by individual non-pathogenic isolates.

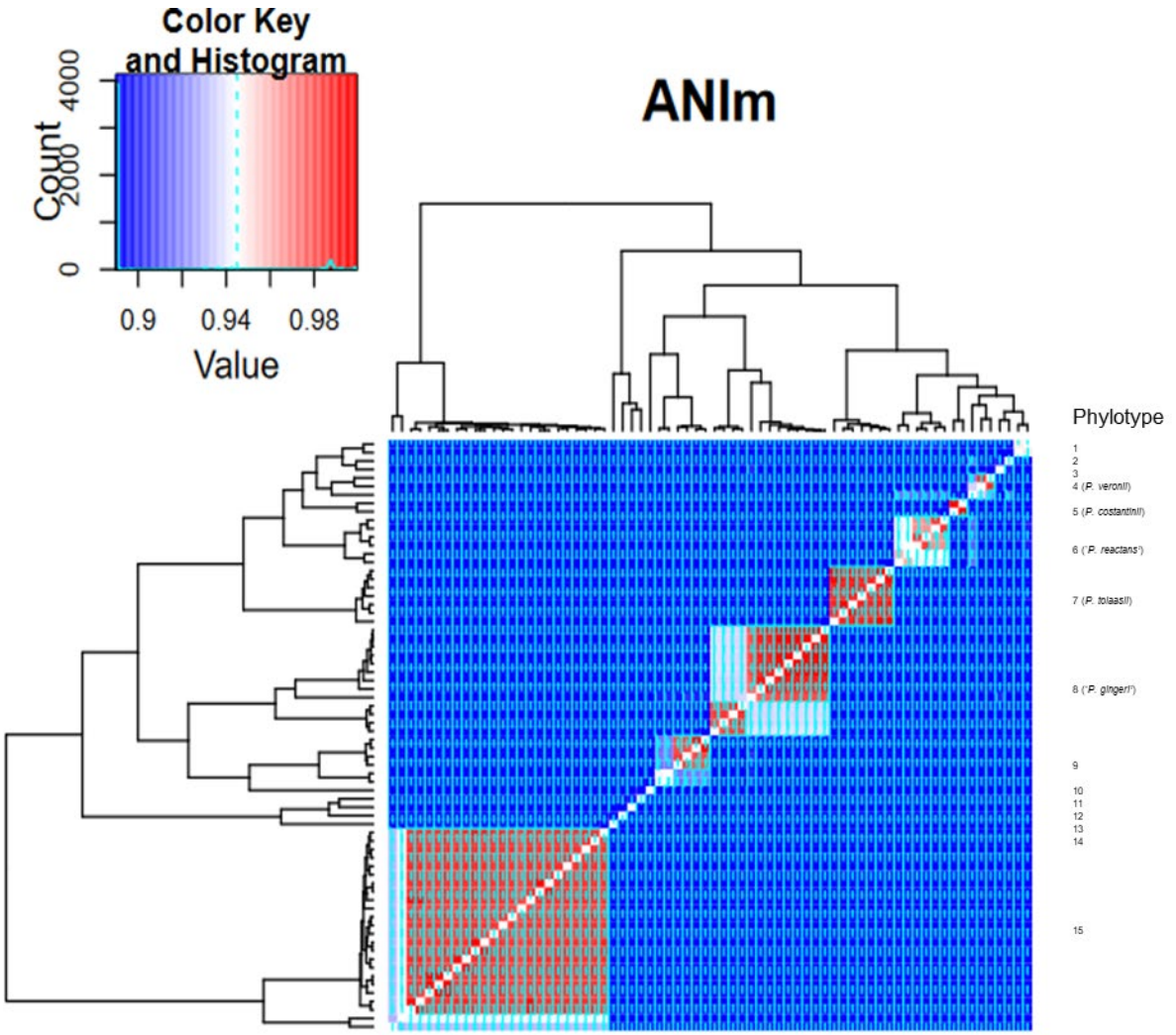


Figure 1. Average nucleotide identity (ANI) analysis comparing genome sequence obtained from recent blotch isolates in the UK, Netherlands and Belgium and reference isolates of fluorescent *Pseudomonas* spp.

Phylotype	Strains analysed*
1	**NCPPB 3637 (CFBP 4832)**; B6002
2	D2002
3	B7002
4	**P7772 (HRI Mar-12)**; **P7771 (HRI WB-1)**; D1002
5	LMG 22119T (= CFBP 5705T); BS2773
6	**P7787**; **P8021**; **NCPPB 3149 (LMG 5329)**; **P7759 (ATCC 14340)**; **P7774 (HRI n-12)**; C5002
7	NCPPB 2192T (P7544); PMS117; 6264; **ATCC 51310 (P7757)**; B1001; A9001
8	NCPPB 3146 (LMG 5327); **P8018**; **21614711**; D1001; C1001; D8001; D5001; C3001; C4002; E1001; B9002; C2001; A6001
9	**P7786**; H7001; H7001b; A8002
10	**P7765 (HRI S. Lincoln T2-6)**; F1001; G9001
11	C2002
12	**P7760 (HRI CH-6)**
13	C7002
14	**P7753**
15	**ATCC 51311 (CFBP 5116)**; **ATCC 51312 (CFBP 5114)**; **21615526**; G5001; D4002; E6002; D6002; B6001; G1002; F8002; D3002b; I8001; C8002; D5002; D5002b; C6002; F2002; A4002; F3002; F9001; D4002b; F1002; B4002; A5002

*Isolates in **blue** were sequenced in this study. Isolates obtained from WUR, Wageningen, Netherlands are pre-fixed A-I followed by a 4 figure number.

2.4. Selection of DNA sequences that (i) are unique to pathogenic isolates and not found in non-pathogenic *Pseudomonas* species (ii) distinguish isolates causing typical bacterial blotch, ginger blotch or mild blotch symptoms.

Genome sequence analysis confirmed that the primer and probe sequences previously designed for *P. tolaasii* detection were found only in the genomes of phylotype 7 isolates that caused severe brown blotch symptoms in the pathogenicity tests (NCPPB 2192, ATCC 51309 and ATCC 51310). This was confirmed by TaqMan analysis of DNA from the purified isolates using the Ptol assay (Table 6). The sequences were also confirmed to be present in the genomes of two additional *P. tolaasii* reference strains (PMS117 and 6264) obtained from the public sequence databases. Interestingly, the isolate NCPPB 2193, which has also been previously assigned to *P. tolaasii,* was found to lack the Ptol sequences, tested negative in the TaqMan assay and was non-pathogenic in the pathogenicity tests.

Similarly, primer and probe sequences for the two '*P. gingeri'*-specific TaqMan assays (Pg2 and Pg6) developed at WUR were found in the genomes of all three UK phylotype 8 isolates that caused severe ginger blotch symptoms in the pathogenicity tests and this was confirmed by TaqMan analysis of DNA from the purified isolates (Table 6). These sequences were also confirmed to be present in the genome of the phylotype 8 reference isolate NCPPB 3146, which also tested positive with the Pg2 and Pg6 TaqMan assays, but did not induce symptoms in the pathogenicity tests, even though this isolate was pathogenic at the time of its deposit into the collection in 1981 when it was proposed as a type strain for '*P. gingeri*'.

The genomes of *P. costantinii* NCPPB 4616 (which caused mild brown blotch) and a further reference strain of *P. costantinii* (BS2773) also lacked any of the TaqMan sequences and NCPPB 4616 was not detected by the TaqMan assays (Table 6). Similarly, one phylotype 9 UK isolate (*Pseudomonas sp.* P7786), which caused only mild ginger blotch symptoms, was not detected by any of the TaqMan assays.

With the exception of NCPPB 3146, none of the non-pathogenic *Pseudomonas* isolates from mushrooms were detected by any of the TaqMan assays.

Table 6. Detection of pathogenic and non-pathogenic *Pseudomonas* isolates using TaqMan assays with designed specificity to *P. tolaasii* and '*P. gingeri*'.

Isolate of fluorescent *Pseudomonas* species					TaqMan results (C_T values)						Pathogenicity test	
					P. gingeri' assays				*P. tolaasii* assay			
Protect	NCPPB	Other	Identity originally assigned	Phylotype	Pg2		Pg 6		Ptol		pot test	cap test
P7548	3637	CFBP 4832	*Pseudomonas gingeri*	1	40	40	40	40	40	40	-	-
P7772		HRI mar-12	*Psuedomonas veronii*	4	40	40	40	40	40	40	-	-
P7771		HRI WB1	*Pseudomonas putida*	4	40	40	40	40	40	40	-	-
	4616^T	CFBP 5705^T = LMG 22119	*Pseudomonas costantinii*	5	40	40	40	40	40	40	+	+
	3149	LMG 5329	*Pseudomonas reactans*	6	40	40	40	40	40	40	-	-
	1311		*Pseudomonas reactans*	6	40	40	40	40	40	40	-	-
P7759		ATCC 14340	*Pseudomonas reactans*	6	40	40	40	40	40	40	-	-
P7787			*Pseudomonas* sp.	6	40	40	40	40	40	40	-	-
P8021			*Pseudomonas* sp.	6	40	40	40	40	40	40	-	-
P7774		HRI n12	*Pseudomonas poae*	6	40	40	40	40	40	40	-	-
P7544	2192^T		*Pseudomonas tolaasii*	7	40	40	40	40	**17.79**	**17.23**	++	++
P7756		ATCC 51309	*Pseudomonas tolaasii*	7	40	40	40	40	**17.68**	**18.49**	++	++
P7757		ATCC 51310	*Pseudomonas tolaasii*	7	40	40	40	40	**18.06**	**18.11**	++	++
	3146	LMG 5327	*Pseudomonas gingeri*	8	**18.39**	**18.04**	**17.49**	**17.21**	40	40	-	-
P8018			*Pseudomonas gingeri*	8	**18.42**	**18.39**	**17.09**	**17.41**	40	40	++	++
		21614711	*Pseudomonas gingeri*	8	**17.47**	**17.17**	**16.62**	**16.62**	40	40	++	++
		21615525	*Pseudomonas gingeri*	8	**17.7**	**17.21**	**16.53**	**17.48**	40	40	++	++
P7786			*Pseudomonas* sp.	9	40	40	40	40	40	40	+	+
P7765		HRI S. Lincoln T2/6	*Pseudomonas putida*	12	40	40	40	40	40	40	-	-
P7760		HRI CH6	*Pseudomonas agarici*	13	40	40	40	40	40	40	-	-
P7753		HRI -	*Pseudomonas syringae*	15	40	40	40	40	40	40	-	-
P7758		ATCC 51311 = CFBP 5116	*Pseudomonas gingeri*	16	40	40	40	40	40	40	-	-
P7779		ATCC 51312 = CFBP 5114	*Pseudomonas gingeri*	16	40	40	40	40	40	40	-	-
		21615526	*Pseudomonas gingeri*	16	40	40	40	40	40	40	-	-
	2289^T		*Pseudomonas agarici*		40	40	40	40	40	40	-	-
	4617^T	CFBP 6595^T	*Pseudomonas protegens*		40	40	40	40	40	40	-	-
	2193	LMG 2343	*Pseudomonas tolaasii*		40	40	40	40	40	40	-	-

1 C_T = Cycle threshold (cycle at which a positive PCR signal is first detected). A negative value is represented by C_T=40. C_T falls as the concentration of target DNA in the reaction increases.

2.5. Develop improved diagnostic tests which detect and differentiate pathogenic *Pseudomonas* species in casing, Phase III compost and mushroom samples.

2.5.1. Effect of compounds on *Pseudomonas tolaasii* suspensions.

No effect of compound A or compound B on the growth of *Pseudomonas tolaasii* isolates was detected at specified concentrations in pure cultures (Figures 3 and 4). Above this concentration, a negative effect of both of these compounds on growth of isolates TRF 42 and TRF 59 was detected using PIA agar (Figure 3). With LB agar, no effect of these compounds was detected until a specified concentration was exceeded (Figure 4). At the same concentration, compound B had a more negative effect on growth than compound A.

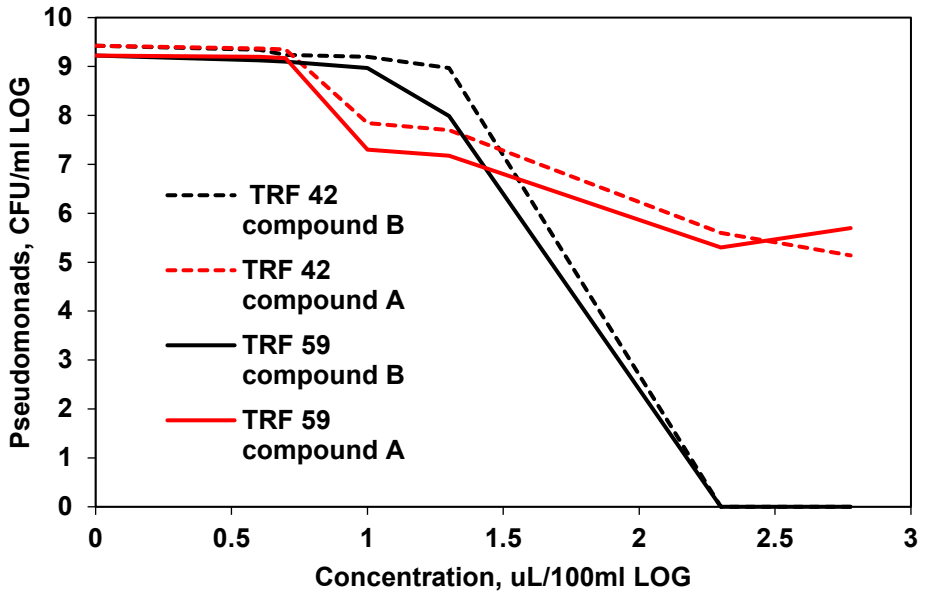


Figure 3. Effect of concentration of compound A and compound B on the growth of *Pseudomonas tolaasii isolates* TRF 42 and TRF 59 determined on *Pseudomonas* isolation agar (PIA). Each value is the mean of four replicates.

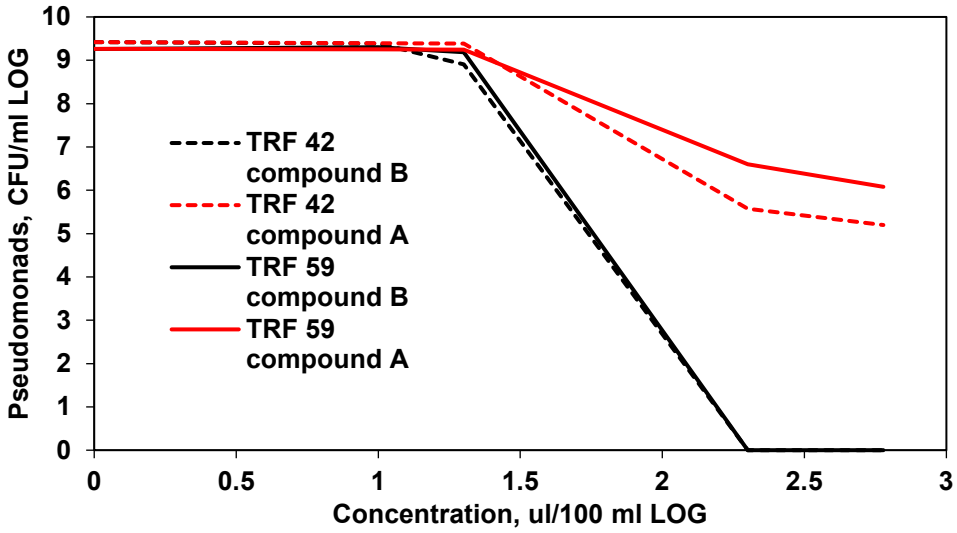


Figure 4. Effect of concentration of compound A and compound B on the growth of *Pseudomonas tolaasii isolates* TRF 42 and TRF 59 determined on LB agar.
Each value is the mean of four replicates.

2.5.2. *Effect of* compounds *on Pseudomonas species in casing samples*

The total population of cultural bacteria in the casing samples, determined in LB agar, was not significantly different between inoculation treatments and ranged between 10^{10} and 10^{11} cfu/g casing (Fig. 5). Differences in the population of *Pseudomonas* species, determined on PIA, were detected between casing samples. *P. gingeri* isolate 24 and *P. tolaasii* isolates 9

and 10 produced lower numbers of cfu than *P. gingeri* isolates 5, 6 and 7 or *P. constatinii* isolate 15 (Figure 5). However, there was no overall difference in *Pseudomonas* populations between casing inoculated with *Pseudomonas* isolates, or uninoculated casing.

Following incubation in compound A or compound B, there were significant increases in the populations of total culturable bacteria and *Pseudomonas* species in the LB broth compared with samples incubated in the control LB broth. For samples inoculated with *P. gingeri* or *P. tolaasii* and incubated with compound A, the increase in *Pseudomonas* population was greater than in casing inoculated with *P. costantinii* or in uninoculated casing samples (Fig. 6). However, there were no significant differences in total culturable bacteria between inoculation treatments that were incubated with compound A or compound B.

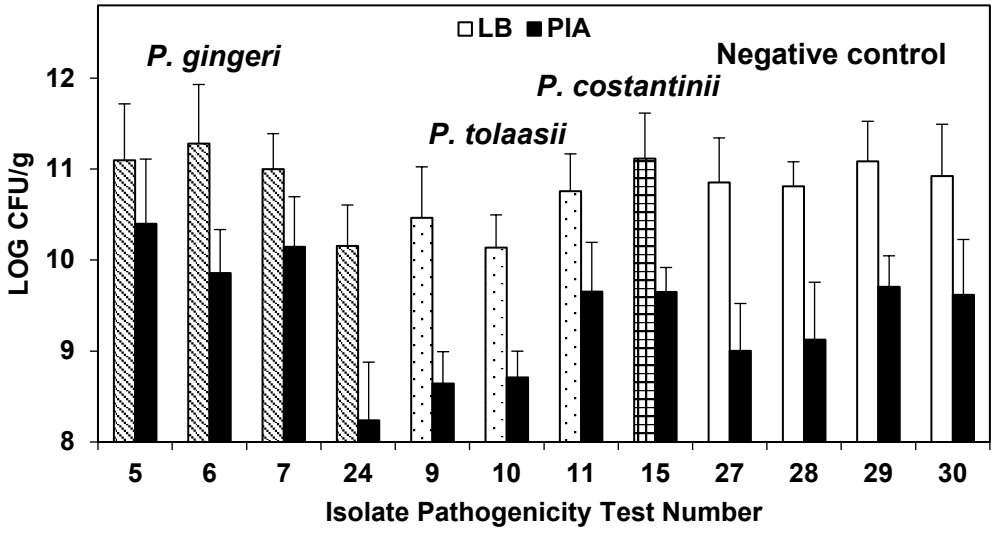


Figure 5. Population in casing samples of total cultural bacteria on LB agar and *Pseudomonas* species on PIA following inoculation of casing with different *Pseudomonas* isolates. Mean of four replicate samples (+SE). LSD (5%) between isolates on the same type of agar = 0.80.

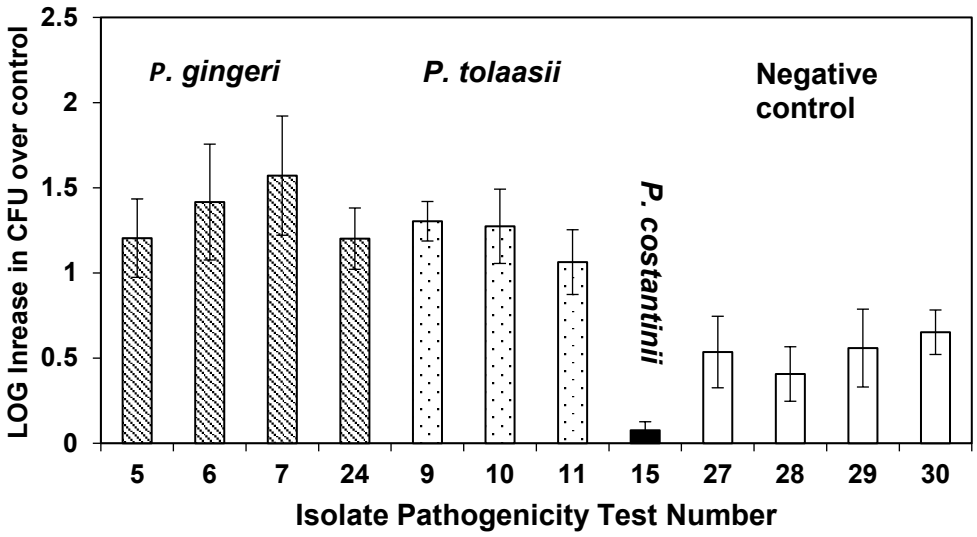


Figure 6. Effect of compound A on increase in *Pseudomonas* species population in samples of inoculated casing samples. Mean of four replicate samples (±SE). LSD (5%) between isolates on the same type of agar = 0.43.

Preliminary results of testing inoculated casing material from the pathogenicity pot tests, using qPCR TaqMan assays for detection of *P. tolaasii* and '*P. gingeri*' before and after enrichment for 16 hrs in LB broth with compound A, are shown in Table 7. Uninoculated casing tested negative with both TaqMan assays. All inoculated samples tested positive for the target pathogen after enrichment, whereas detection in the non-enriched inoculated samples was usually unsuccesful. Interestingly, *P. tolaasii* was also detected in casing that had been inoculated with '*P. gingeri*' isolates and vice versa, indicating possible spread of inoculum between pots during the course of the pathogenicity experiments, which was then detected, especially after enrichment.

Table 7. Cycle threshold (C_T) values obtained after testing DNA extracted from inoculated casing samples with qPCR (TaqMan) assays designed for detection of *Pseudomonas tolaasii* (Ptol-2) or '*P. gingeri*' (Pg6).

Pre-enrichment	Original Inoculum	*P. gingeri* (assay Pg6)		*P.tolaasii* (assay Ptol-2)	
No	*P. gingeri* (21614711)	40.00	40.00	40.00	40.00
Yes		21.18	20.66	34.69	33.42
No	*P. gingeri* (21615525)	40.00	24.86	40.00	40.00
Yes		20.88	21.01	40.00	40.00
No	*P. tolaasii* (NCPPB 2192^T)	40.00	40.00	40.00	40.00
Yes		32.55	40.00	40.00	36.53
No	*P. tolaasii* (ATCC 51310)	26.64	27.07	22.88	22.70
Yes		32.13	32.04	24.69	24.73
No	None	40.00	40.00	40.00	40.00

2.6. Investigate control of different types of bacterial blotch by manipulation of the casing bacterial population.

2.6.1. Bacterial populations in casing

At the end of Experiments 1 and 2, casing populations of total cultivable bacteria (1.58 – 3.50 x 10^8 CFU/g) and *Pseudomonas* spp. (1.25 – 4.75 x 10^7 CFU/g) were not significantly different in treatments (a) to (g).

Casing prepared from the Irish Harte peat had a higher moisture content than the Sterckx casing (Table 8). Casing containing hydrated lime or CI had a lower electrical conductivity than casing containing sugar beet lime or compost caccing.

Table 8. Analysis of casings used in the experiments.

Peat	Lime	Caccing	Moisture, %	pH	EC, µS/cm
(a) Harte	SBL	Compost	86.4	7.69	265
(b) Sterckx	SBL	Compost	81.0	7.61	300
(c) Harte	Hydrated	Compost	84.7	7.76	207
(d) Harte	SBL	CI	86.8	7.63	178

2.6.2. *Mushroom yield and blotch disease incidence*

There were significantly more blotched mushrooms per pot from the high humidity room (91-93% RH) than from the low humidity room (85-87% RH) (Figure 7). Irrigating mushrooms with a $CaCl_2$ solution resulted in significantly fewer blotched mushrooms than irrigating mushrooms with the same volume of water, or the other treatments. This occurred at both the high and low levels of relative humidity. The following treatments had no effect on the number of blotched mushrooms:

- Irish peat (Harte) or German peat (Sterckx)
- Sugar beet lime or hydrated lime
- Phase III compost caccing or casing inoculum (CI)
- Irrigation with water or a glucose solution
- Untreated casing compared with applications of Ultimate Mushroom Boost before the first and second flushes.

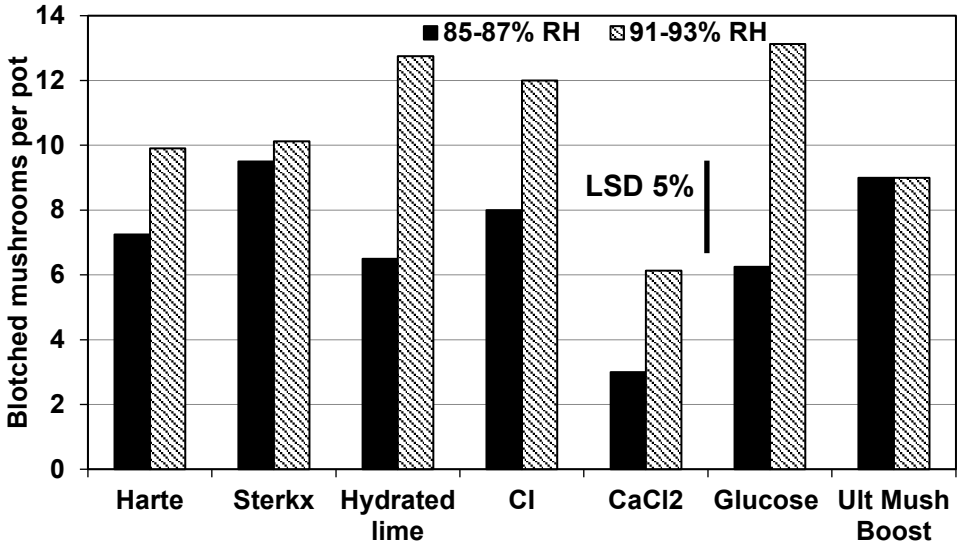


Figure 7. Effect of growing room relative humidity and casing and irrigation treatments on the number of blotched mushrooms. CI: casing inoculum.

There were no significant effects of the growing room humidity or of treatments (a) to (g) on the yield of mushrooms (range of treatment averages 321-367 g/kg compost).

3. Discussion

The literature review and experimental results described above have attempted to clarify the complex taxonomy of the fluorescent *Pseudomonas* species that are major components of the bacterial communities found in mushroom casings. In particular, the subset of these bacteria that is able to cause bacterial blotch symptoms has been characterised. According to whole genome sequence analysis, the cause of recent severe ginger blotch symptoms sampled in the UK in 2011 and 2016 was identified as a single phylotype that was also shared by the reference strain of '*Pseudomonas gingeri*' (NCPPB 3146). This phylotype was not detected by the quantitative TaqMan qPCR test previously developed to the tolaasin gene sequence from *P. tolaasii*. However, it was successfully detected using new TaqMan qPCR assays developed by collaborators at Wageningen Plant Research (WUR) in the Netherlands. Furthermore these assays were designed and confirmed to specifically recognize other strains belonging to the same phylogroup but not the closely-related non-pathogenic *Pseudomonas* species in other phylogroups, which are commonly isolated from mushroom casings. Similarly, the qPCR assays previously designed for detection of *P. tolaasii*, were confirmed to be specific to the single phylotype which comprised reference strains of *P. tolaasii* and other related isolates causing severe brown blotch. As a result, it is now possible to detect the two main causes of severe bacterial blotch; *P. tolaasii*, the cause of severe

brown blotch and '*P. gingeri*' the cause of severe ginger blotch, using a combined approach with the two assays.

An extended analysis included genome sequences obtained from additional *Pseudomonas* isolates collected by collaborators at WUR from recent mushroom blotch outbreaks on commercial production sites in the Netherlands and Belgium. Strains causing severe brown and ginger blotch were also assigned to the same *P. tolaasii* and '*P. gingeri*' phylotypes as isolates from the UK. However, pathogenicity tests identified other isolates from the UK and Netherlands/Belgium collections that were able to induce blotch symptoms. These strains were allocated to another 2 phylogroups (9 and15) and were not detected by the qPCR tests that have been developed. Furthermore, the reported ability of a strain of *P. costantinii* to cause mild brown blotch in Finland (Munsch *et al*., 2002) was confirmed and this strain was allocated to a further phylogroup (5). *P. costantinii* was not, however, detected amongst isolates from the recent blotch outbreaks studied. The importance of these other blotch causing strains, in terms of the actual losses they incur during commercial production and the efficacy of current control measures, requires further investigation. If necessary, it will now be possible to develop additional qPCR tests from the available genomic sequences that were used to differentiate them.

In an effort to improve the sensitivity of diagnostic methods to detect pathogenic *Pseudomonas* species in casing materials, Phase III composts and mushroom samples, experiments were conducted to investigate the feasibility of pre-enriching populations of the target organisms following treatment with compounds. Results showed that compound A had a greater stimulatory effect than compound B on the casing population of *Pseudomonas* species, are in agreement with Noble *et al.* (2009). Preliminary results have shown that pre-treatment of inoculated casing samples with compound A increased the likelihood of detecting *P. tolassii* and '*P. gingeri*' using the available TaqMan qPCR assays. Further controlled investigations will be required to assess the suitability of this approach for screening of casing materials and phase III composts to eliminate sources of contamination with these pathogens from commercial production.

Finally, further investigation on the control of different types of bacterial blotch by manipulation of the casing bacterial population has been described. The reduction in blotch resulting from addition of 0.3% $CaCl_2$ to the irrigation water is in agreement with the findings of Alpuche-Solis and Paredes-Lopez (2001). Previous work has also shown that $CaCl_2$ in the irrigation water can reduce bacterial spoilage post-harvest (Solomon *et al*., 1991). $CaCl_2$ is not approved for use in the EU; however, it is used in irrigation water in some countries (e.g. USA, Australia and New Zealand) as a 'whitening agent'. It may be possible to detect a higher

chloride level in mushrooms treated with $CaCl_2$; however, this would also occur in mushrooms growing in casing materials with a higher chloride content, for example spent mushroom compost. Further work is needed on how $CaCl_2$ supresses bacterial blotch on mushroom caps e.g. through increasing the osmotic pressure of the solution on the mushroom cap, or by leaving a hygroscopic layer of $CaCl_2$ on the mushroom cap surface. This may either inhbit bacterial growth or increase the resistance of the mushroom hyphae to rupture. It is not clear whether a similar disease suppressive effect could be achieved by using a very dilute salt (NaCl) solution or other calcium or chloride salt solutions. Salt is approved for use on mushroom crops (Anon. 2017).

4. Conclusions

1. Causes of severe mushroom blotch in the UK were previously identified as *Pseudomonas tolaasii* and '*Pseudomonas gingeri*', causing severe brown blotch and severe ginger blotch respectively.
2. Phylotypes of other *Pseudomonas* strains that can cause mild blotch symptoms, or that are non-pathogenic to mushroom were differentiated at the species level from *P. tolaasii* and '*P. gingeri*' by whole genome analyses.
3. Surveys of commercial mushroom production in the Netherlands and Belgium have found the same species of bacteria causing severe blotch as in the UK, although other, as yet unnamed, species are also suspected to be able to cause severe blotch.
4. There was good agreement between the results of pot culture and cut cap tissue bioassays in terms of pathogenicity of *Pseudomonas* isolates.
5. Molecular tests using quantitative real time (TaqMan) polymerase chain reaction were identified that detected either the *P. tolaasii* or '*P. gingeri*' strains that cause severe blotch but did not cross react with other non-pathogenic *Pseudomonas* strains or strains that cause mild blotch symptoms.
6. Genome sequence of mild blotch-causing *Pseudomonas* phylotypes is available for potential further diagnostic development if they are found to be commercially important.
7. Incubation of casing samples in LB broth containing compound A or compound B resulted in a greater pseudomonad population than incubation of casing samples in LB broth alone.

8. Incubation of mushroom casing samples in LB broth containing compound A resulted in a selectively greater increase in the *Pseudomonas* population when *P. tolaasii* or '*P. gingeri*' were present in the samples.

9. Irrigation of mushrooms with a 0.3% $CaCl_2$ solution resulted in significantly fewer blotched mushrooms than irrigating with the same volume of water, both at high and low levels of relative humidty.

10. There was no difference in the level of blotched mushrooms between several other casing treatments tested.

11. There were no significant effects of the growing room humidity or of treatments (a) to (g) on the yield of mushrooms (range of treatment averages 321-367 g/kg compost).

12. The populations of total cultural bacteria and of *Pseudomonas* in the casing was not affected by irrigation with a 0.3% $CaCl_2$ solution or by the other casing treatments used.

5. Recommendations for Further Work

1. The blotch control efficacy of irrigating mushrooms with $CaCl_2$ solution should be compared with other Ca^{++} or Cl^- solutions. Salt (NaCl) which is registered as a commodity substance or hydrated/milk of lime ($Ca(OH)_2$) which is used as a casing material may be easier to apply (from a regulatory perspective) in the short term. Irrigation with dilute pasteurised spent compost or casing leachate or 'tea' (which is high in KCl) may also be effective in suppressing blotch.

2. The enrichment of *Pseudomonas* populations in casing samples using compound A to improve the detection limit of subsequent real time TaqMan PCR analysis requires further investigation.

3. The economic significance of mild blotch causing *Pseudomonas* strains observed in the UK and of other blotch-causing *Pseudomonas* species observed in the Netherlands and Belgium requires further investigation. This should also include further investigation of reported strains assigned to the *P. syringae* species complex and *P. aeruginosa*, which have been found in mushroom culture and to produce tolaasins.

4. Newly available genome sequence data should be exploited for further development of TaqMan assays for detection of any other important blotch causing *Pseudomonas* species, in addition to those now developed for *P. tolaasii* and '*P. gingeri*'.

5. A wider survey of UK mushroom production would allow the importance of blotch causing species other than *P. tolaasii* and '*P. gingeri*' to be assessed.

6. Knowledge and Technology Transfer

- Results from this project will be presented at the AHDB Mushroom Conference 2019 on 25th April 2019 at Woodside, Glasshouse Lane, Kenilworth, CV8 2AL.
- A joint scientific publication of the genome sequencing and pathogenicity work is planned together with collaborators J. van der Wolf and T. Taparia at Wageningen Plant Research (WUR) in the Netherlands.
- The review article (Appendix 1) is also intended for separate scientific publication.

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Appendix

Diversity of pathogenic and beneficial fluorescent *Pseudomonas* species affecting cultivated mushroom production: A review.

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Introduction

Mushroom blotch diseases have been described for over a century (Tolaas, 1915; Paine, 1919). The symptoms of blotch were reviewed by Kobayashi and Crouch (2009). Blotch symptoms typically occur on basidiocarps (or caps) and occasionally on mushroom stipes. They begin initially as a lesion of discolouration, which eventually become sunken or pitted at later stages, depending on the type of blotch disease. Two blotch diseases, brown blotch and ginger blotch, have been described and can be distinguished from each other based on the colour and physical nature of the damage observed on the basidiocarps. The diseases differ primarily by the colour intensity of symptoms produced, which has led to some confusion presented in the literature as to the causative agents and specific diseases. Classic brown blotch symptoms are characterized by a dark brown discoloration that eventually appears as deep sunken lesions. In contrast, ginger blotch symptoms typically have a lighter, ginger-coloured appearance, with lesions showing no or only mild sunken appearance (Wong *et al.*, 1981). Severe pitting can also occur, but it is not clear whether this is caused by the same bacterial species (Beyer 2005; Fletcher & Gaze, 2007). Because it is apparent that severity of blotch disease symptoms can vary, especially under varying environmental conditions, correct diagnosis can be difficult. Furthermore, studies have shown that differences in colour can be species and strain dependent (Wells *et al.*, 1996; Soler-Rivas *et al*., 1999; Godfrey *et al*., 2001). Blotch disease-causing bacteria are readily found in soil and casing material used for mushroom cultivation (Wells *et al*., 1996; Godfrey *et al*., 2001b), suggesting that blotch diseases occur due to the activity of opportunistic saprophytic bacteria.

The development of consistently effective management strategies for bacterial blotch in cultivated mushrooms requires a better understanding of the genetic diversity of the pathogenic blotch-causing *Pseudomonas* species. This will assist the selection of improved pathogen detection methods and disease control measures which avoid unwanted negative

impacts on populations of closely related beneficial pseudomonads, which stimulate initiation of mushroom pinning and may also play a role in suppressing pathogenic populations.

Pseudomonas species causing bacterial blotch disease of cultivated mushrooms

Bacterial blotch is caused by a diverse group of bacteria in the genus *Pseudomonas* (Wells *et al*., 1996; Yamamoto *et al*., 2000; Godfrey *et al.*, 2001a and 2001b; Abou-Zeid, M. A. 2012; Yun *et al*., 2013). Variation in symptom severity and incidence and inconsistencies and overlaps in the names assigned to blotch-causing bacteria have frequently led to confusion as to the causative agents of the various types of blotch.

Brown blotch is frequently caused by metabolically diverse strains of *Pseudomonas tolaasii* in the *P. fluorescens* species complex (Wells *et al*., 1996; Munsch *et al*., 2000; Soler-Rivas *et al*., 1999; Godfrey *et al.*, 2001a and 2001b; Munsch *et al*., 2002a and 2002b) that produce tolaasin, a cyclic lipopeptide (CLP) toxin that is essential for induction of disease symptoms by these bacteria (Brodey *et al*., 1991; Rainey *et al*., 1991). *P. tolaasii* strains differ in the types of CLP molecules they produce (Bassarello *et al*., 2004). Furthermore, CLP toxins are produced by other diverse bacteria from the *Pseudomonas fluorescens* species complex that cause brown blotch symptoms similar to those caused by *P. tolaasii* (Nielsen *et al.*, 2000; de Brujin *et al*., 2007). These include *Pseudomonas costantinii* (Munsch *et al.*, 2002) and *Pseudomonas fluorescens* strain NZ17 (Godfrey *et al*., 2001a). Munsch and Alatossava (2002a) showed that all pseudomonads producing tolaasins were haemolytic, inducing lysis of sheep or bovine erythrocytes. However, production of various tolaasins has also been reported in isolates from the *Pseudomonas syringae* species complex (Lazzaroni *et al*., 2003) and in an isolate identified as *Pseudomonas aeruginosa* (NCPPB 2195), although its pathogenicity on mushroom was not determined. *P. aeruginosa* has been obtained from unused and used mushroom casing and mushrooms (Morgan *et al*. 1999).

Ginger blotch has been attributed to a group of strains in the *P. fluorescens* species complex referred to as '*Pseudomonas gingeri'* (Wong *et al.*, 1981; Cutri *et al.*, 1984) although this is not considered a taxonomically-valid species name. Further investigation is needed to determine whether ginger blotch is caused by a genetically distinct group of bacteria, or whether it results from the effect of environmental variation on symptom intensity.

Non-tolaasin-producing bacteria are also capable of causing blotch disease, although symptoms are often less intensive. These include strains referred to as '*Pseudomonas reactans*' (Iacobellis and Lo Cantore, 2003a and 2003b), which produce a related lipodepsipeptide compound known as white line inducing principle (WLIP). '*P. reactans*' is not considered a taxonomically valid species name. Production of a white line in culture media between colonies of *P. tolaasii* and '*P. reactans*', due to reaction and precipitation between

the two lipopeptides, was formerly used to identify *P. tolaasii* isolates (Wong and Preece, 1979), although other related bacteria are now known to induce the same reaction (Munsch and Alatossava, 2002b). '*P. gingeri*' does not give a positive reaction in the WLIP test (Goor *et al*. 1986). Neither '*P. reactans'* nor WLIP toxin cause blotch symptoms at the same intensity or severity as *P. tolaasii* or tolaasin (Lo Contore and Iacobellis, 2004). WLIP-producing strains have been identified in both *Pseudomonas fluorescens* and *P. putida* species complexes (Rokni-Zadeh *et al.*., 2013a).

Other blotch-causing bacteria include a strain of *Pseudomonas protegens* (Pf-5), which causes symptoms through a lipopeptide-independent mechanism involving the production of 2,4-diacetylphloroglucinol and pyoluteorin that induced blotch symptoms when applied as the purified compounds (Henkels *et al*., 2014). *P. protegens* is better known as a producer of secondary metabolites and a plant disease biocontrol agent (Haas and Keel, 2003; Loper and Gross, 2007; Gross and Loper, 2009; Sonnleitner and Haas, 2011). *Pseudomonas agarici* is also included among the bacterial species reported to be associated with causing milder blotch-like symptoms on *Agaricus bisporus* (Geels *et al*., 1994; Godfrey *et al*., 2001b; Lo Cantore and Iacobellis, 2004) and has also been identified as the causative agent of yellow blotch disease of oyster mushrooms, which is characterized as yellow droplets on the surface of sporocarps (Bessette *et al*., 1985) and bacterial blotch of king oyster mushroom (Rodriguez-Estrada & Royse 2006). *P. agarici* is also well known as the causative agent of drippy gill (Gill and Cole, 2000). This disease is characterized by bacterial ooze originating from gills of sporocarps and from longitudinal splits within the stipe where bacteria colonized host tissue both intercellularly and intracellularly. Unlike the brown blotch pathogen, virulence factors involved with *P. agarici* pathogenesis have not been established.

Recent work (Sepulveda *et al*., 2017) compared *Pseudomonas* isolates from symptomatic mushrooms in different production farms in the USA using repetitive element PCR (rep-PCR) and multi-locus sequence analysis (MLSA). As many as seventeen different genotypes belonging to at least six different species were isolated. Similar studies in the Netherlands (Van der Wolf *et al*., 2016) found three species (so far reported as *P. tolaasii*, '*P. gingeri*' and an unnamed species) able to cause severe blotch symptoms as well as other unnamed species able to cause mild symptoms. The cause of recent serious outbreaks of ginger blotch in the UK has yet to be determined. Isolates from some mushrooms with ginger blotch symptoms did not give a positive reaction when tested with PCR assays designed to detect tolaasin gene DNA from *P. tolaasii* (Lee *et al.*, 2002; Elphinstone, 2012) and further investigation is needed to identify the cause of ginger blotch symptoms in these outbreaks.

Interaction between pathogenic and beneficial fluorescent *Pseudomonas* spp.

Agaricus bisporus production requires a casing layer, which has imprecisely defined chemical and microbiological properties that stimulate primordium formation (Fermor *et al.*, 2000; Noble *et al*., 2009; Colauto *et al.*, 2016). The presence of stimulatory bacteria in the casing, of which *Pseudomonas putida* is regarded the most significant, is usually necessary for primordium formation to occur (Rainey, 1989). Like *P. tolaasii*, *P. putida* is positively attracted toward exudates of mushroom mycelium and rapid and firm adherence of these bacteria to hyphae has been observed. During the preliminary phase of the interaction between *P. putida* and *A. bisporus*, the bacterium was shown to markedly affect hyphal growth, colony morphology and the allocation of resources to the mycelium. Mutants of *P. putida* defective in their ability to promote primordium initiation were defective in their ability to uptake a ferric-siderophore complex, implicating the involvement of iron regulated, membrane bound protein porins in the process of fruit body initiation. In addition to *P. putida*, other *Pseudomonas* spp. with stimulatory activity have also been identified, including *P. poae* and *P. veronii* (Noble *et al.*, 2009). Fructification does not occur under axenic cultivation in a sterile casing layer but can be induced under such conditions by addition of sterile active charcoal or large numbers of stimulatory pseudomonads (Fermor *et al.*, 2000). Certain volatile organic compounds in casing, mainly C8 compounds including 1-octen-3-ol and 2-ethyl-1-hexanol, are known to inhibit primordium formation and are metabolized by stimulatory pseudomonads and removed by active charcoal (Noble *et al.*, 2009). Removal of organic volatiles from casing by ventilation also enables primordium formation to occur under axenic conditions.

Colauto *et al.* (2016) showed that different casing materials contained 10^7-10^9 cfu per gram dry weight of bacteria, of which 75-85% were identified as *Pseudomonas* spp. and 12% as *P. putida*. Control of pathogen blotch-causing pseudomonads in casing material therefore involves a delicate balance between suppression of pathogenic strains whilst maintaining a healthy balance of beneficial *Pseudomonas* species. Hence, chemical or physical sterilization of casing material to remove pathogen populations is only a viable option if beneficial organisms can be replaced through inoculation or primordium formation can be controlled by removing inhibitory volatiles. Fermor *et al.* (1991) screened over 100 fluorescent *Pseudomonas* spp., isolated from mushroom farms, which were potentially antagonistic to *P. tolaasii*. Inoculation of selected antagonists (at $2x10^{11}$ cfu per m^2) onto commercial mushroom beds on three occasions (casing, pinning and post first flush) consistently reduced bacterial blotch by at least 50%. Ozactan and Bora (1994) also demonstrated inhibition of bacterial blotch using fluorescent pseudomonads.

A more recent promising strategy to control pathogenic strains of fluorescent pseudomonads without affecting beneficial strains is to target them with specific lytic bacteriophage. Various

phages with specific activity against *P. tolaasii* have been described (Munsch and Olivier, 1993; Kim et al.., 2011; Nguyen *et al*., 2012: Sajben-Nagy *et al.*, 2012). Current UK research is investigating the level of control offered by a cocktail of different phage isolates produced by APS Biocontrol Ltd. (Angus, Scotland) during commercial mushroom production. Success will depend on selection and maintenance of a phage cocktail with a sufficiently broad spectrum of activity against the full range of blotch-causing pseudomonads.

Taxonomic relatedness between pathogenic and beneficial fluorescent *Pseudomonas* spp.

The genus *Pseudomonas* currently contains 144 species that are recognised in the List of Prokaryotic Names with Standing in Nomenclature (Parte, 2014). Phylogenetic analysis of mushroom blotch-causing pseudomonads on the basis of 16S rRNA gene sequence diversity (Anzai *et al.*, 2000; Godfrey *et al.*, 2001a and 2001b) revealed high homogeneity of 16SrRNA sequence across the range of fluorescent pseudomonads but also indicated the diversity of species able to induce blotch symptoms on *Agaricus bisporus*. A clearer phylogeny between *Pseudomonas* species has recently been established using multilocus sequence analysis (MLSA) of housekeeping genes (Yamamoto *et al.*, 2000; Tayeb *et al.*, 2005; Sajben *et al.*, 2011; Gomila *et al.*, 2015). Whole genome comparisons are also available for many representatives of the species, but not yet for all type strains (Gomila *et al.*, 2015). Genomic analysis of 112 *Pseudomonas* strains showed a continuous gradient of genetic relatedness throughout the genome. Within species, MLSA values are at least 97% similar and average nucleotide identity (ANI) values are at least 95% similar (Mulet *et al.*, 2010 and 2012; Konstantinidis and Tiedje, 2005; Richter and Rosselló-Móra, 2009). On these bases, the *Pseudomonas* genus divides into phylogenetic groups, each of which represents a complex of existing *Pseudomonas* species and may in turn be subdivided into different subgroups or lineages (Yamamoto *et al.*., 2000, Gomila *et al.*, 2015. Some additional species, including *P. agarici*, do not conform to any of the 11 phylogenetic groups established by Gomila *et al.* (2015).

Most of the blotch-forming pathogens are located within the *Pseudomonas fluorescens* phylogroup, with the exception of blotch-causing strains of *P. agarici* (Geels *et al.*, 1994; Godfrey *et al.*, 2001b; Lo Cantore and Iacobellis, 2004). Of eight sub-groups defined within the *P. fluorescens* phylogroup, *P. tolaasii* and *P. costantinii* isolates cluster within the *P. fluorescens* sub-group, whereas the blotch-causing strains *Pseudomonas protegens* Pf-5 and *P. fluorescens* NZ17 group within the *P. chlororaphis* sub-group (Gomila *et al.*, 2015). A growing database of whole genome sequence is becoming available for blotch-causing and closely related strains (Silby *et al.*, 2009; Loper *et al.*, 2012; Ghequire *et al.*, 2013; de Lima-Morales *et al*., 2013; Rokni-Zadeh *et al*., 2013b: Montes *et al*., 2016; van der Wolf *et al.*,

2016). Further comparative genomic studies are required to better resolve the identification of pathogenic and beneficial *Pseudomonas* spp. and to develop more accurate methods for their detection leading to a better understanding of virulence factors and the environmental conditions that promote or suppress disease.

Detailed phylogenetic analysis of the *P. putida* species complex has also relied on MLSA analysis of housekeeping genes (Yamamoto and Harayama, 1998; Yamamoto *et al.*, 2000; Mulet *et al.*, 2013; Gomila *et al.*, 2015; Yonezuka *et al.*, 2017) and has also been confirmed by matrix-assisted laser desorption/ionization-mass spectrometry of ribosomal proteins (MALDI-MS) (Teramoto *et al.*, 2007). Many strains originally classified as *P. putida* biovar B were found to require reclassification since they clustered in various subgroups of the *P. fluorescens* phylogroup rather than with the *P. putida* biovar A strains in the *P. putida* species complex, which includes the type strain of *P. putida* (ATCC 12633). Most recently Yonezuka *et al*. (2017), have revealed the taxonomic diversity of 59 strains identified as *P. putida,* which were classified into 26 species-level clades. Further investigation of this type is needed to enable differentiation of beneficial and pathogenic strains of *P. putida* isolated from mushroom casings.

Bacterial Blotch Control

Antagonist Pseudomonads

Bacterial antagonists (non-pathogenic pseudomonads) have been identified for blotch control in mushrooms (Nair & Fahy, 1972 and 1976; Fahy *et al.*, 1981). The work resulted in an Australian biocontrol product, Conquer, and a product in the USA, Victus, based on the *Pseudomonas fluorescens* biovar V strain NCIB 12089. These products have been reported to give good control of blotch (Miller 1993; Miller & Spear 1995) but none is currently marketed. Olivier and Guillaumes (1983), Fermor *et al*. (1990) and Bora & Ozaktan (2000) also used non-pathogenic *Pseudomonas fluorescens* isolates, obtained in France, the UK and Turkey respectively, for controlling bacterial blotch. Saxon *et al*. (2014) found that the bacterium *Bdellovibrio bacteriovorus* protected mushrooms from brown blotch caused by *P. tolaasii*.

Noble & Dobrovin-Pennington (2017) examined the use of *Pseudomonas chlororaphis* and *Bacillus subtilis* for control of fungal diseases in mushrooms. They did not cause blotch under cropping conditions that were not conducive to the disease; the effect of these bacterial biocontrol agents on blotch disease was not examined.

Humidity and Air Flow Control

The conditions which encourage blotch are associated with moisture, such as the persistence of water on basidiocarps for more than three hours (Gandy, 1985). Edwards (1978) used a table of 'coefficients of evaporation' (air velocity × saturation deficiency) for determining the conditions for producing healthy mushrooms, where the saturation deficiency in mb was determined from the air temperature and relative humidity (RH), and the air velocity was measured in m/min. Values of <4 were likely to cause blotch and >10 likely to cause scaling. Edwards (1979) later indicated the acceptable range for the coefficient of evaporation to be 15-30. However, subsequent tests by Sabeh *et al*. (2005) showed that scaling was more prevalent than blotch at <15 while little scaling occurred in a room with >30. It is not clear whether a high relative humidity and air velocity has the same effect on blotch and mushroom cropping as a low relative humidity and air velocity (for the same air temperature and coefficient of evaporation). Lomax (2007) showed that it is dew point temperature that is critical in determining whether water will condense on mushroom caps and increase the risk of blotch. Fluctuations in air temperature in a growing room can result in the cap temperature being below the dew point temperature. He suggested keeping the RH below 90% to keep the mushroom caps dry. Hermans (1989) recommended an RH of 80-85% during growth of fruit-bodies.

Casing and compost materials and caccing

Wong and Preece (1980) found that casing was the primary source of *P. tolaasii* on mushroom farms although less than 2% of samples tested positive. One of the positive peat samples was obtained from a peat bog remote from mushroom farms. The presence of mushroom tissue was essential for the establishment of the bacterium and *P. tolaasii* inoculated into casing disappeared in the absence of *A. bisporus*. Olivier (1982) could not reliably isolate *P. tolaasii* from casing materials. Elphinstone *et al*. (2013) could identify *P. tolaasii* in fresh casing material using a Taqman PCR *Pseudomonas tolaasii* test but other blotch-causing *Pseudomonas* species could not be detected. Fahy *et al.* (1981) and Olivier (1982) found no relationship between blotch and different casing materials. However, Olivier (1982) was able to isolate *P. tolaasii* from spawn-running compost and the addition of colonised compost to casing increased the occurrence of blotch in some but not all instances. Spear *et al*. (1994) also found evidence for increased blotch following the use of spawn-run compost as caccing material instead of sterile casing inoculum.

Resistant mushroom strains

None of the commercial strains of *A. bisporus* is resistant to blotch although brown and cream strains are more tolerant than white strains (Olivier 1982).

Chemicals

The most widely used bactericides have been sodium hypochlorite (Ayers & Lambert, 1955) and chlorine (Miller & Spear, 1995; Fletcher & Gaze, 2007). Sodium hypochlorite products normally contain 10-12% available chlorine. It is the hypochlorous acid content which is most bactericidal; the dissociation of hypochlorite into hypochlorous acid or chlorine is pH dependent, the optimum is pH 6.5. Chlorine was added to irrigation water at 150 ppm (Fletcher & Gaze, 2007). Chlorine and hypochlorite become rapidly inactivated causing variable results when used on crops susceptible to blotch (Gandy, 1985). A solution of chlorine dioxide, activated by citric acid, was found to give control of bacterial blotch (Geels *et al*., 1991). Neither chlorine, sodium hypochlorite nor chlorine dioxide are registered for use in the UK. An acetic acid solution (70-80 mM) has been effective against bacterial yellowing of king oyster mushroom (*Pleurotus eryngii*) (Bruno *et al*., 2014) so might be effective in blotch disease control in *Agaricus bisporus*.

Calcium chloride (0.3%) and hydrogen peroxide (0.75%) added to irrigation water significantly reduced postharvest bacterial browning (Chiktimmah *et al*., 2006). The use of 0.3% calcium chloride in irrigation water has been shown to reduce bacterial populations and the incidence of blotch (Alpuche-Solis *et al*., 2001). Ozone has been used for controlling *Pseudomonas aeruginosa* in water supplies (e.g. Puradigm Technology, Las Vegas, NV www.puradigm.com) but has not been tested on mushrooms.

Disinfectants

A 15 second exposure to isopropanol (70%), bleach (a.i. sodium hypochlorite, 1:9 dilution), Premisan (a.i. organic potassium salts, 1:250 dilution) or OxiDate (a.i. hydrogen peroxide, 1:100 dilution) was effective in destroying the activity of *Pseudomonas tolassii*. A one-minute exposure to Verticide (a.i. dodecyl dimethyl ammonium chloride, 1:250 dilution) or Environ (a.i. phenolic compounds, 1:250 dilution) was also effective (Romaine *et al*., 2003).

Conclusions

1. Bacterial blotch is caused by a diverse group of bacteria in the genus *Pseudomonas*. Variation in symptom severity/incidence and inconsistencies and overlaps in the names assigned to blotch-causing bacteria have frequently led to confusion as to the causative agents of the various types of blotch.
2. Severe brown blotch is mostly caused by bacteria in the *Pseudomonas fluorescens* species complex, which produce toxic peptides known as tolaasins that contribute to symptom development. These include *Pseudomonas tolaasii*, mostly known as the causative agent of severe brown blotch. Other tolaasin-producing strains in the *P.*

fluorescens species complex that can also cause severe blotch symptoms, have been assigned to the species *P. costantinii* and *P. fluorescens*.

3. Tolaasins have also been found to be produced by strains of bacteria assigned to the *P. syringae* species complex and *P. aeruginosa*, although the pathogenicity of these strains requires confirmation.
4. Another group of strains in the *Pseudomonas fluorescens* species complex have been invalidly named as '*Pseudomonas gingeri'*, on the basis that they cause ginger blotch symptoms. Outbreaks of severe ginger blotch have recently been observed in Europe. Diagnostic tests based on detection of specific tolaasin gene sequences do not appear to detect '*P. gingeri*' strains.
5. Mild blotch symptoms can also be caused by bacteria that are not known to produce tolaasin toxins. Such strains have been assigned to *P. protegens*, the invalidly-named species '*P. reactans'* and *P. agarici* (better known as the cause of drippy gill disease of mushrooms).
6. Phylogenetic analysis in the USA and the Netherlands has shown significant diversity amongst *Pseudomonas* isolates from mushrooms with blotch symptoms. The degree to which this diversity relates to the current nomenclature within known blotch-causing species requires further clarification. Further investigation is also needed to confirm the pathogenicity of the different phylotypes identified and to relate these to current outbreaks of mushroom blotch so that appropriate detection tests and control strategies can be developed.
7. Non-pathogenic *Pseudomonas* species present in mushroom casing are important because they stimulate primordium formation. The most significant of these have been assigned to *P. putida,* although other *Pseudomonas* species may also be involved. However, these strains are genetically diverse, and many strains originally classified as *P. putida* biovar B (including the type strain of *P. putida*) have been reclassified into the *P. fluorescens* species complex. Further investigation is required to be able to correctly identify these beneficial bacteria and to differentiate them from the pathogenic *Pseudomonas* species.
8. Some non-pathogenic *Pseudomonas fluorescens* strains have been shown to have biocontrol activity against the blotch-causing bacteria. A strain of *Bdellovibrio bacteriovorus* also protected mushrooms from brown blotch caused by *P. tolaasii*. However, no commercial biocontrol products are currently available.
9. Current blotch controls rely on controlling humidity (<90%) and air flow to avoid condensation of water on the mushroom cap in which the pathogenic bacteria can multiply, whilst avoiding scaling that occurs when the humidity is too low. However, there are some contradictions in the relative humidities and air flows which have been shown

to cause blotch and scaling. Avoiding fluctuations in temperature and humidity, and the resulting condensation on mushrooms, may be as or more important than maintaining specific constant values.

10. Improvements to the sensitivity and specificity of detection methods are needed so that casing and caccing materials can be reliably tested for the presence of low populations of the full range of pathogenic *Pseudomonas* species and thus eliminate them as potential primary sources of infection.
11. Efficacy of a number of disinfectants against *P. tolaasii* has been proven. These include 70% isopropanol, 10% sodium hypochlorite bleach, organic potassium salts (Premisan), hydrogen peroxide (OxiDate), dodecyl dimethyl ammonium chloride (Verticide) and phenolic compounds (Environ).
12. Experimental chlorination of irrigation water using chlorine, sodium hypochlorite or citric acid-activated has reduced bacterial blotch by various degrees but none of these are registered for use on mushrooms in the UK. Other water treatments with potential to reduce blotch include 70-80 mM acetic acid, 0.3% calcium chloride, 0.75% hydrogen peroxide and ozonation.

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