Burkholderia cepacia complex bacteria and their antimicrobial activity

Thesis presented for the degree of Philosophiae Doctor

By

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Summary

Background. *Burkholderia cepacia* complex (Bcc) are well known for their ability to produce antifungal agents. However, the majority of past studies have screened only a limited number of isolates, which were not taxonomically well characterized. In addition, while the activity of Bcc bacteria against plant pathogenic fungal species has been characterized, very few studies have examined the interaction between Bcc and saprotrophic wood decay basidiomycete species. The overall aim of this study was to begin to the characterization of the interactions of Bcc and other *Burkholderia* with the model ascomycete *Candida albicans* and woodland basidiomycete fungi.

Methods. The study made use of a large collection (397 isolates) of taxonomically well identified *Burkholderia* species. A systematic screening of the antifungal activity of this collection was made against *C. albicans* and various wood decay basidiomycetes: *Bjerkandera adusta, Trametes versicolor, Hypholoma fasciculare, Resinicium bicolor* and *Phanerochaete velutina*. A wide range of media were evaluated for *Burkholderia* growth, antifungal metabolite production and the ability to support the mycelial growth of basidiomycetes. An agar overlay assay was used to examine the anti-candidal activity of *Burkholderia* isolates, while a novel overlay assay was developed using homogenised mycelial material for the basidiomycete species. Novel and existing extraction techniques were evaluated for their ability to partially purify active *Burkholderia* metabolites. The purification and identification of the active *Burkholderia* antibiotics were performed using both preparative thin layer chromatography (TLC) and liquid chromatography combined with mass spectrometry (LC-MS). A novel TLC-bioautography assay was also developed to reveal the presence of active *Burkholderia* metabolites in the antimicrobial extracts. The minimum inhibitory concentration (MIC) of the semi-purified *Burkholderia* antifungals was also determined. The natural diversity of *Burkholderia* and other bacteria associated with different rhizosphere environments was evaluated by cultivating isolates from local maize crops and examining a collection of *Burkholderia* isolates obtained from the rainforest in Sabah, Malaysia. The genetic basis for the secretion of antifungal agents by *B. ambifaria* was examined using transposon mutagenesis and screening for the mutants which had lost their anti-candidal activity. Additional transposon mutants that had lost their anti-bacterial activity and had been obtained in a previous screen were also examined for alterations in antifungal activity. Finally, the *Burkholderia* genome database and bioinformatic techniques were used to genetically characterize these mutants and other genetic loci that had been previously implicated in antibiotic production.

Results. The majority of *Burkholderia* isolates possessed the same antagonistic activity towards *C. albicans* as they did towards *B. adusta*. Of the 397 isolates examined, antifungal activity was the greatest in the following *Burkholderia* species: *B. ambifaria*, *B. cepacia*, *B. cenocepacia* and *B. contaminans*. Interestingly, no antifungal activity was observed for *B. multivorans* (27 isolates) and *B. stabilis* (18 isolates) under the

experimental conditions tested. The type of medium used to study the interaction between *Burkholderia* and the fungi considerably effected antifungal activity, with the most suitable media for studying antagonistic activity being Sabouraud agar and an acidic minimal salts medium (BSM; pH 6) supplemented with 0.4% glycerol. Of the 397 isolates screened, 47.85% were anticandidal (190 isolates; 89.47% of these were Bcc species and 10.53% were other *Burkholderia* species), while 48.10% were active against *B. adusta* (191 isolates; 93.19% of these were Bcc species and 6.81% were other *Burkholderia*). Extraction using Amberlite XAD16 resin binding followed by elution with methanol was an excellent means to isolate active *Burkholderia* antifungals. Fractionation of these extracts using silica gel TLC and ethyl-acetate:methanol:water (20:1:0.5) as the elution solvent was optimal for the separation of the majority of the active metabolites. A TLC-bioautography assay was developed in this current study as a preliminary screening technique to detect anti-microbial components of novel *Burkholderia* extracts. The assay was very useful for detecting anti-candidal and anti-bacterial compounds and was adapted for use for the first time with basidiomycetes. Antifungal *Burkholderia* isolates produced between 1 and 10 antifungal metabolites. Several antifungal agents were identified as enacyloxin, pyrrolnitrin, bactobolin and quinolines. Novel compounds and novel bactobolin derivatives were also present. For *B. ambifaria* strain AMMD a number of chromosomal loci were found to be involved in the production of antifungal metabolites. Quorum sensing was essential for the expression of antifungal activity as transposon mutations in the CepI synthase-encoding gene prevented the biosynthesis of all *B. ambifaria* anti-candidal compounds. *B. ambifaria* AMMD extracts containing enacyloxin IIa also produced an inhibitory effect at low concentration against *E. coli*, *B. multivorans* and *A. baumannii*.

Conclusions. *Burkholderia* are potentially a very large reservoir of known and novel antimicrobial agents, especially with nearly 50% of isolates screened exhibiting antifungal activity. Antimicrobial compounds such as enacyloxin IIa were discovered for the first time to be produced by *Burkholderi* and novel extraction and bioassay methods for anti-fungals were developed in this study. Overall, we now have the tools to considerably advance the isolation and characterization of antifungal *Burkholderia* metabolites.

Declaration

This work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree.

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Dedication

I dedicate this study in loving memory to my late father Belkasem Boaisha who encouraged me tirelessly to complete my study in a successful manner; to my lovely mother Fatimah Sultan who also continuously supported me in my studies, and finally to the brave Libyan revolutionaries who spent their souls in 17 of February 2011 for Libyan freedom dignity and happiness.

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LIST OF ABBREVIATIONS

µg	**Microgram**
ACT	**Artemis Comparison Tool**
AfcA	**Lipopeptide**
AMMD	**An isolate of *Burkholderia ambifaria***
ATCC	**American typing collection center**
Bamb	***B. ambifaria* gene-blast**
Bcc	***Burkholderia cepacia* complex bacteria**
BCF	**An isolate of *Burkholderia ambifaria***
BGD	***Burkholderia* Genomes Database**
BK1	***Bjerkandera adusta* an isolate of wood decay basidiomycetes**
BLAST	**Basic Local Alignment Search Tool**
Bp	**Base pair**
BSM	**Basal salts medium**
BSM-G	**Basal salts medium-glycerol**
CAM	**Casein meat agar medium**
CDS	**Coding Sequence**
CF	**Cystic fibrosis**
Cfu	**Colony forming units**
COG	**Clusters of Orthologous Groups**
D2	***Trametes versicolor* an isolate of wood decay basidiomycetes**
DNA	**Deoxyribonucleic acid**
EPA	**environmental protection agency**
GC%	**Guanine cytosine content**
HAQ	**4-hydroxyl-3-ethyl-2-alkyl-quinoline**
HFDD2	***Hypholoma fasciculare* an isolate of wood decay basidiomycetes**
HFGTWV2	***H. fasciculare* an isolate of wood decay basidiomycetes isolate**
HMAQ	**4-hydroxyl-3-methyl-2-alkyl-quinoline**
HQA	**Quinolinone**

HSL	**N-octanoyl-homoserine lactone**
ISOA	**Isosensitest agar medium**
JW13	**An isolate of *Burkholderia vietnamiensis***
kb	**Kilo bases (1kb = 1000 bases)**
Kc311	**An isolate of *Burkholderia ambifaria***
LC	**Liquid chromatography**
MA	**Malt agar medium**
Mb	**Mega bases (1Mb = 1,000,000 bases)**
MeOH	**Methanol**
MHA	**Muller Hinton agar medium**
ml	**Millilitre**
MS	**Mass spectra**
NCBI	**National center for biotechnology information**
NCTC	**National Collection of Type Culture**
ND	**Not detected**
nm	**Nanometre**
OD	**Optical density**
OR	**Open Reading Frame**
PCR	**Polymerase chain reaction**
PDA	**Potato dextrose agar medium**
PEG	**polyethylene glycol**
pH	**Hydrogen ion concentration**
PIJHYA	***Phallus impudicus* an isolate of wood decay basidiomycetes**
PKS	**Polyketide synthase**
Prn	**Pyrrolnitrin gene**
Prn	**Pyrrolnitrin**
PV29	***Phanerochaete velutina* an isolate of wood decay basidiomycetes**
QS	**Quorum sensing**
RAPD	**Random amplified polymorphic DNA**
Rb1	***Resinicium bicolor* an isolate of wood decay basidiomycetes**

Rbm6A	***Resinicium bicolor* an isolate of wood decay basidiomycetes**
Rf	**Retention factor**
RNA	**Ribonucleic acid**
S.E	**Standard error of the mean**
SA	**Sabouraud agar medium**
Sa1	***Stereum gausapatum* an isolate of wood decay basidiomycetes**
SP313	***Heterobasidion annosum* an isolate of wood decay basidiomycetes**
TAE	**Tris Acetic acid EDTA buffer**
TE	**Tris-EDTA**
TLC	**Thin layer chromatography**
Tn	**Transposon**
TSB, TSA	**Tryptone Soya broth/ agar medium**
TTC	**Triphenyl tetrazolium chloride**
UV	**Ultraviolet light**
YE	**Yeast extract**

CHAPTER ONE

GENERAL INTRODUCTION AND AIMS

General introduction

The search for novel microbial metabolites has been motivated by the need for new antibiotics to combat microbial multidrug-resistance to current antimicrobial agents. Microorganisms are an important source of potentially useful metabolites. Up to now, bacterial species of the genus *Streptomyces* and fungal species of the genus *Penicillium* have mainly been investigated and used for the isolation of antibiotics. *Burkholderia* are soil bacteria able to use a wide range of organic compounds and produce a wide range of potent antimicrobial agents (Parke & Gurian-Sherman, 2001, Vial*, et al.*, 2008, Mahenthiralingam*, et al.*, 2011). Nearly all of *Burkholderia* antibiotics characterised to date have been identified from *B. cepacia* isolates which have not been characterized at the species level in terms of the *B. cepacia* complex (Bcc) taxonomic group (Mahenthiralingam*, et al.*, 2008). Further, no studies have looked at the antimicrobial activity within large collections of taxonomically-defined Bcc isolates. *Burkholderia* should be investigated in more detail as producers of antimicrobial metabolites and potentially developed as new sources of clinically useful antibiotics.

1.1 Interaction between bacteria and fungi

Bacteria and fungi are evolutionarily very distinct microorganisms. However, they often live in close contact with each other, regularly share common substrates, and their spatial proximity in many environments has led to either synergistic or antagonistic interactions between them. The biosynthesis of natural products by microorganisms is affected by biotic and abiotic factors. Each microorganism has the ability to produce particular metabolites through distinctive mechanisms. In the natural environment the growth

substrates and conditions may vary considerably and alter the metabolites secreted by microorganisms. In the laboratory, the components of synthetic media and different factors such as pH, temperature and incubation period have been shown to alter antibiotic biosynthesis in many producer strains (Kadir*, et al.*, 2008). For detailed chemical characterization and commercial production it is necessary to optimize the antibiotic production conditions (Duffy & Defago, 1999, Raaijmakers*, et al.*, 2002, Kadir*, et al.*, 2008). Understanding these production conditions in the laboratory will also provide means to explore what antimicrobial agents are produced in nature by known biological control organisms such as *Burkholderia*.

The interaction between bacteria and fungi, especially pathogenic species, has been studied by many researchers for long time, but limited attention has been given to interactions between wood decay basidiomycetes and bacteria. There are many of *B. cepacia* complex (Bcc) strains known to be antimicrobial producers and suggested as bio-pesticides (Parke & Gurian-Sherman, 2001). Bcc strains designated as type Wisconsin and proposed as bio-pesticides were submitted to US Environmental Protection Agency (EPA) for registration in the late 1980s. Many Bcc bio-pesticides were registered between 1992 and 1996. Later, polyphasic taxonomic studies and genetic analysis demonstrated clear relationships between Bcc bio-pesticidal and clinical strains. These results led to consideration of the hazards of continued use of Bcc as bio-pesticides and (EPA) cancelled registration and prohibited the use of Bcc bio-pesticides (Parke & Gurian-Sherman, 2001).

The interaction between bacteria and wood decay basidiomycetes ranges from highly competitive to mutulistic. Antagonistic interactions can be mediated via volatile or

diffusible antibiotics or following contact (Boddy, 2000). Many fungi acidify culture media during growth by production of organic acids (Petrson & Mclennan, 1948, Espejo & Agosin, 1991), and this might be expected to be inhibitory to bacteria even in the absence of other antibiotics (Tsuneda & Thorn, 1995). Attack of bacteria by wood decay basidiomycetes and their use as a source of nutrients is a well-known phenomenon (Tsuneda & Thorn, 1995, Elsas*, et al.*, 2007). The most aggressive bacterial attackers are often the fast growing wood decay basidiomycetes (Tsuneda & Thorn, 1995).

Currently, the control of phytopathogenic fungi depends heavily on the use of synthetic chemicals as primary control agents in agriculture. However, the interest in biological control has steadily increased for a number of reasons such as the costs of chemical fungicides, concerns over their environmental impact, public worry about the hazards associated with these chemicals, safety problems and development of resistance by certain plant pathogens (Weller, 1988, Whipps, 2001, Elsas*, et al.*, 2007).

1.2 *Burkholderia*

Burkholderia species were originally recognized as phytopathogenic bacteria which cause sour skin rot of onion bulbs (Burkholder, 1950). *Burkholderia* are β proteobacteria, Gram negative, mostly motile, aerobic, morphologically straight or slightly curved bacilli (1 to 5 μm in length and 0.5 to 1.0 μm in width). They do not form spores and their optimal growth temperature ranges between 20ºC and 37ºC. They are able to utilize a variety of simple and complex carbohydrates including alcohols, amino acids (Vermis*, et al.*, 2003) and even hydrocarbon fuels (O'Sullivan & Mahenthiralingam, 2005) as carbon sources. The genus *Burkholderia* contains more than 40 different species, which inhabit a large

array of ecological niches. They exist in soil, water, plant rhizosphere, endophytically and are associated with fungi (Parke & Gurian-Sherman, 2001).

1.2.1 *Burkholderia cepacia* complex

Burkholderia cepacia complex (Bcc) is a group of closely related species. These species share a high degree of 16S rRNA gene (98 to 100%) and *rec*A gene (94 to 95%) sequence similarity (Vermis, *et al.*, 2003). They all also hold unusually complex and plastic genomes that consist of two or more chromosomal replicons which harbour multiple insertion sequences (Mahenthiralingam & Vandamme, 2005).

Bcc were recognised as opportunistic human pathogenic bacteria responsible for a variety of infections, but only in individuals with an underlying vulnerability. Bcc bacteria are very problematic in cystic fibrosis (CF) patients because of their native resistance to a wide range of antimicrobial agents and their ability to spread from patient to patient (Tablan, *et al.*, 1985). Also within the genus *Burkholderia* is *B. mallei,* the causative agent of glanders in horses, mules and donkeys, and *B. pseudomallei,* the causative agent of melioidosis, an infectious disease of humans and animals in tropical areas (Berriatua, *et al.*, 2001, Coenye & Vandamme, 2007).

1.2.2 The diversity of *Burkholderia*

Bcc are ecologically versatile bacteria able to metabolise a wide range of carbon compounds. They are also one of the dominant bacterial groups found in the plant rhizosphere. Bcc bacteria have also been isolated from diseased plants, such as onion (Burkholder, 1950, Saddler, 1994), ryegrass (Nijhuis, *et al.*, 1993), maize (Dalmastri, *et*

al., 1999), pea (King & Parke, 1996), cotton (Hallmann*, et al.*, 1999), coffee (Estrada*, et al.*, 2001), sugar-cane (Bramer*, et al.*, 2001) and papaya (Kadir*, et al.*, 2008).

Microbial rhizosphere community structure and density may be affected by biotic factors (eg. other microorganisms and their metabolites, and animal and plant populations) and abiotic factors (eg. temperature, water content or pH). The diversity of the culturable *Burkholderia* communities in rhizosphere soils is affected by several factors. Di Cello*, et al.*, (1997) suggested that the diversity and density of the *B. cepacia* community associated with maize-root depended on plant growth stage and root exudates. Dalmastri*, et al.*, (1999) found that the type of the soil was the main factor which affected the diversity of the *B. cepacia* population associated with maize-roots.

Burkholderia species have also been shown to occur in symbiosis with fungi. Yara *et al*. (2006) described the presence of a bacterium belonging to Bcc associated with Brazilin white-rot fungus *Pleurotus* sp. Lim *et al*. (2003) characterized and proposed the *B. sordidicola* KCTC^{T} 12081 and KCTC 12082 as a novel species which was associated with fungus *Pleurotus sordid*, collected from the plants *Quercus acutissium* and *Prunus serrulata.*

Bacteria are often found attached to fungal hyphae, spores and within fruiting bodies. A range of species including *Pseudomonas*, *Bacillus*, *Paenibacillus* and *Burkholderia* have already been found to have a connection with fungi (Elsas*, et al.*, 2007). The fungus may provide both nutrients and colonization sites for the bacteria and the bacteria may also attack and use fungi for their own nutrition, or they may produce antibiotics that inhibit or

kill fungi (Highley & Dashek, 1998).

Nearly all species within the Bcc have been isolated from both the clinical and environmental isolates, however, isolates within the Cardiff collection (> 1600 isolates of *Burkholderia* species) have an interesting distribution (Mahenthiralingam*, et al.*, 2008). Environmental isolates were not detectable within the *B. cenocepacia* III D and only a few environmental isolates of *B. multivorans, B. cenocepacia* III A, *B. dolosa and B. stabilis* were present in the collection (Mahenthiralingam*, et al.*, 2008). All 16 isolates of *B. cenocepacia* IIIC were environmental isolates. Moreover, for *B. ambifaria, B. pyrrocinia*, unclassified species groups BCC5 and BCC6, more than 89% of isolates were from natural environments (Mahenthiralingam*, et al.*, 2008). Although such collections of bacteria help with understanding the diversity of environments that *Burkholderia* bacteria can be cultivated from, further studies based on prospective collection of isolates or cultivation-independent analyses are needed to fully explore sources of these bacteria in nature.

1.3 Fungi

Fungi play a major role in the function and dynamics of ecosystems. Fungal mycorhiza with plant root are crucial for host nutrient acquisition. Fungi are the main agents of decomposition and hence of nutrient release. Fungi may also cause serious economic problems in agriculture. Several important plant and human pathogenic fungi such as *Fusarium*, *Rhizoctonia*, *Cryptococcus* and *Candida* spp have been used as target organisms screened for the discovery of new antifungal substances. In this study, ten representatives of wood decay basidiomycetes and *C. albicans* were used as target fungal species in order to screen for antifungal biosynthesis by *Burkholderia* bacteria.

The Basidiomycota comprises a large phylum containing about 22,000 known species. Some basidiomycetes are facultative parasites, for example *Armillaria* sp causes root rot. Smuts and rusts are obligate parasites causing plant diseases that cause damage worth millions of pounds yearly. Saprotrophic basidiomycetes are the major agents of wood decomposition in forest soils and form about 60% of living microbial biomass (Boddy*, et al.*, 2008). Wood rots are categorized either as white rots, brown rots or soft rots. White rots attack and destroys all compounds of wood including lignin, cellulose and hemicellulose. Brown rots attack and destroy cellulose and hemicellulose but not lignin. Soft rot operate a more local scale, and cause different patterns of decay, and again do not utilize lignin (Highley & Dashek, 1998). Basidiomycetes are known sources for the production of a large number of secondary metabolites. For example they produce a wide range of potent extracellular enzymes such as lignin peroxidase, glyoxal oxidase and laccase (Boddy, 2000), and are also able to produce antibiotics (de Boer*, et al.*, 2008).

Chemically synthesised or petrochemical industry-derived wood preservatives have been banned in several countries because of both health concerns and environmental issues. Thus, there is an urgent need for new approaches to preserving wood and preventing fungal wood decay (Highley & Dashek, 1998). Secondary metabolites produced by microorganisms in the field of biocontrol, especially from *B. cepacia* complex (Bcc) have shown promising activity as anti-fungal agents (Rousk*, et al.*, 2008).

C. albicans belongs to Ascomycota and the family Saccharomycetaceae. It is commonly found as part of the normal microflora on the skin and orifices of humans. It is the major opportunistic fungal pathogen causing potentially fatal disease in immunosuppressed

human hosts (Pfaller & Diekema, 2007). Since *C. albicans* is a very common cause of fungal infections in humans, it has been a major target species for the discovery of new anti-fungals.

1.4 Biological control

Biological control agents (BCAs) are those microorganisms which can be used to reduce pathogens or to decrease damage caused by them (Elsas*, et al.*, 2007). This is of increasing importance because of the cost of pesticides, legislation against their use, and increasing immunity to them. Biological control of soilborne pathogens has been studied for over 85 years. Bacterial biocontrol agents improve plant development by suppressing pathogens (Weller, 1988). Biocontrol may also provide control of diseases that cannot be controlled by other approaches (Cook, 1993).

Microbes that can grow in the rhizosphere are often used as biocontrol agents. Many different bacterial genera and species have been studied. Many studies have used *Bacillus, Pseudomonas*, *Agrobacterium*, *Streptomyces*, *Alcaigenes*, *Enterobacter*, *Erwinia*, *Xanthomonas* and *Burkholderia* as biocontrol agents (Barea*, et al.*, 2005). Pathogen suppression by antagonistic microorganisms occurs via different mechanisms such as parasitism, competition for sites of colonization, carbon, nitrogen and iron sources, and inhibition of the pathogen by antimicrobial agents (Whipps, 2001, Barea*, et al.*, 2005), secretion of bacteriocins (Fravel, 1988), and volatile organic compounds (Cartwright*, et al.*, 1995, Wheatley, 2002). Several microorganisms attack pathogens by excreting cell wall hydrolysis enzymes such as chitinase and β-1,3-glucanase. Fridlender *et al*. (1993) showed that *B. cepacia* produces β-1,3-glucanase that can destroy *Rhizoctonia solani,*

Sclerotium rolfsii and *Pythium ultimum* cell walls.

1.4.1 Fungi as biological control agents

There are a range of different types of interaction between fungi and bacteria, including mutualism, parasitism, competition or predation. Among the fungi, many species have antagonistic properties and have been applied in biocontrol, such as *Trichoderma* species. *Trichoderma* spp have been widely researched and have potential in control of wood decay by fungi. Some fungi, such as *Pleurotus ostreatus* and *Agaricus brunnescens* attack bacteria such as *Pseudomonas* and *Agrobacterium,* lysing them by secreting specific enzymes and using them as a source of nutrients. The white rot fungi *Hypholoma fasciculare* and *Resinicium bicolor* have bactericidal and bacteriostatic properties, which may involve in the production of toxic compounds (Folman*, et al.*, 2008). Such interactions may give the fungi a considerable advantage in obtaining nutrients in different environments (Elsas*, et al.*, 2007).

Ejechi (1998) studied the interaction between four species of wood decay fungi, *Gloeophyllum sepiarium, Gloeophyllum* sp., *Pleurotus* sp. and *Trametes* sp., and two ureolytic bacteria, *Bacillus* sp. and *Proteus* sp., on artificial media and wood. The biomass of wood decay fungi was clearly reduced in a medium containing urea, as a result of bacterial activity. In contrast, the biomass of wood decay fungi was not affected by the presence of urelytic bacteria in non-urea containing media. The bacteria produced volatiles which reduced the growth of wood decay fungi.

Murray and Woodward (2003) investigated the effects of bacteria, isolated from stumps of Sitka spruce, on the ability of wood decay fungi (*Heterobasidion annosum*, *Hypholoma fasciculare*, *Resinicium bicolor, Melanotus proteus, Sistotrema brinkmannii,* and *S. sanguinolentum*) to grow and cause wood decay. Inoculating wood with these bacteria 10 d after inoculation with *H. annosum* resulted in a highly significant decrease in weight. The interaction between different fungi and these bacteria on agar and broth media fluctuated depending on the medium type. Bacterial isolates had a greatest inhibitory effect on *H. annosum* when grown on sporulation agar and yeast dextrose peptone agar compared with corn meal agar and NLM liquid medium.

Folman *et al*. (2008) studied the effect of two white rot fungi, *H. fasciculare* and *R. bicolor* on community composition and the number of bacteria colonizing sterile beech wood. Their results suggested that the white rot fungi reduced the number of bacteria occupy beech wood and the composition of the bacterial community was also altered. Bacteria belonging to 18 families were detected in wood samples and soil beneath colonized wood and fungal mycelium. *Burkholderiacae* were dominant in the un-colonized and basidiomycete colonized beech wood. *Xanthomonasaceae* were also prevalent species in un-colonized beech wood and in *H. fasciculare* colonized beech wood.

1.4.2 *Burkholderia* as biological control agents

Bcc strains designated as type Wisconsin and at the time known as "*Pseudomonas cepacia*" were registered as bio-pesticides in United States and obtained EPA approval in the late 1980s. Available data at that time indicated that clinical and environmental strains had several different phenotypic properties. Several Bcc bio-pesticides were registered

between 1992 and 1996. Polyphasic taxonomic studies and genetic markers later revealed no key differences between Bcc bio-pesticidal and clinical strains. These results led to consideration of the hazards of continued use of Bcc as bio-pesticides and EPA cancelled registration and prohibited use Bcc bio-pesticides (Parke & Gurian-Sherman, 2001). Sequence-based typing methods such as multilocus sequence typing demonstrated that Bcc strains from environmental and clinical sources may be clonal (genetically identical) (Baldwin*, et al.*, 2008).

Despite the current moratorium on the registration of Bcc biopesticide strains, several studies have shown that they are highly potent biocontrol species. Heungens and Parke (2000) studied biocontrol activity of *B. cepacia* strain AMMDR1 against two Oomycete pathogens of pea, *Pythium aphanidermatum* and *Aphanomyces euteiches.* AMMDR1 exhibited strong biocontrol activity against both of these fungus like species, and in particular protected pea and sweet corn seeds from *Pythium* damping-off disease . Li *et al*. (2002) illustrated an aggressive effect of *B. ambifaria* isolate BCF against *Pythium ultimum* that caused cucumber and soybean damping-off disease, and reported broad spectrum antifungal activity against *P. ultimum, R. solani, P. capsici* and *F. oxysporum* . Chul-Hoon *et al*. (2004) showed a strong inhibitory effect of a *B. ambifaria* isolate against both *Erysiphe graminis* and *Puccinia recondite,* the causative agents of barley powdery mildew and wheat leaf rust, respectively.

1.4.2.1 *Burkholderia* antimicrobial agents

Antimicrobial agents are a chemically heterogeneous group of organic, low molecular weight compounds produced by microorganisms that, at low concentrations, inhibit the

growth or metabolic activity of other microorganisms (Duffy, *et al.*, 2003). Antimicrobial agents play an important part in the disease suppression by bacteria and fungi (Weller, 1988). Various antibiotics have been isolated from both fungal and bacterial species.

There are many Bcc species that produce metabolites with antifungal activity (Table 1.1). These include pyrrolnitrin (Cartwright, *et al.*, 1995), altericidins (Kirinuki, *et al.*, 1977), cepacin A and cepacin B (Parker, *et al.*, 1984), pseudoanes and cepacidines A and B (Meyers, *et al.*, 1987), pseudane (Homma, *et al.*, 1989), phenazine (Cartwright, *et al.*, 1995), cepaciamides A and B (Jiao, *et al.*, 1996), quinolinone (Moon, *et al.*, 1996), lipo-peptides and AFC-BC11 (Kang, *et al.*, 1998), and CF661(Quan, *et al.*, 2006). Examples of well characterized Bcc antibiotics are described in Table 1.1.

1.4.2.1.1 Pyrrolnitrin

Pyrrolnitrin (Figure 1.1) is a chlorine-containing phenylpyrrole derivative, with strong antifungal activity that is produced by Gram negative bacteria (Loper & Gross, 2007). It was first discovered in a "*Pseudomonas cepacia*" strain that was subsequently reclassified as the Bcc species *B. pyrrocinia* (Vandamme, *et al.*, 2002). Other Gram negative bacteria are also known for their ability to produce pyrrolnitrin (Arima, *et al.*, 1964, Cartwright, *et al.*, 1995) and it has now been found to be produced by several Bcc species (Schmidt, *et al.*, 2009).

Table 1.1 List of known Bcc isolates with their reported antifungal agents and susceptible fungi

Antimicrobial agents	Bcc strain	Susceptible fungi (tested against)	Author
Quinoline	*P. cepacia* strain PCII	-	Moon *et al*. 1996
AFC-BC11 (Lipo-peptides)	*B. cepacia* isolate BC11	*R. solani*	Kang *et al*. 1998
Altericidins	*P. cepacia*	*Alternaria kikuchiana*	Kirinuki *et al*. 1977
Cepaciamids A and B			Jiao *et al*. 1996
Cepacin A and B			Parker *et al*. 1984
CF66I	*B. cepacia* strain CF-66	*Colletorichum lindemuthianum*, *R. solani*, *A. alternate, Bipolanis sorokiniana, Curvularia lunata* and *Monochaetia hirta*	Quan *et al*. 2006
		C. albicans and *F. solani*	Li *et al*. 2007, 2008
Phenazine	*B. cepacia* strain 5.5B	*R. solani*	Cartwright *et al*. 1995
Pseudane	*B. cepacia* strain RB425	*Pyricular oryza*, *R. solani* and *Verticillium dahliae*	Homma *et al*. 1988
Pseudoanes			Meyers *et al*. 1987; Lee *et al*. 1994
Pyrrolnitrin	*B. cepacia* strain RB425	*P. oryza, V. dahliae* and *R. solani.*	Arima *et al.* 1964
	B. cepacia isolate B37w	*Fusarium sambucinum*	Homma *et al*. 1989
	B. cepacia strain 5.5B	*R. solani*	Burkhead *et al*. 1994
	B. cepacia NB-I		Cartwright *et al*. 1995
	B. cepacia strain 5.5B and RR 21-2	*Trichoderma pseudokonigii*	Roitman *et al*. 1990
	B. cepacia isolates J2535, LMG 1222 and ATCC 51671		El-Banna and Winkelmann 1998
		C. gloeosporioids	Kadir *et al*. 2008
	B. cepacia strain B23		Sultan *et al*. 2008

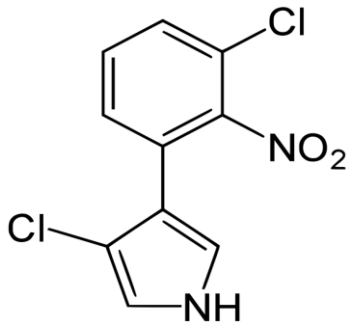


Figure1. 1 The structure of pyrrolnitrin

Several studies have confirmed that pyrrolnitrin produced by Bcc has a broad spectrum activity that includes Gram-positive bacteria such as *Streptomyces* species. However, it is well characterized in terms of its antifungal activity against a wide range of basidiomycetes and ascomycetes, including several economically important pathogens e.g. *Fusarium sambucinum, R. solani, Botrytis cinerea* and *Sclerotinia sclerotiorum* (Burkhead*, et al.*, 1994, El-Banna & Winkelmann, 1998, Ligon*, et al.*, 2000). Homma, *et al*., (1988) showed that *B. cepacia* strain RB425, which was isolated from a lettuce root, produced three different kinds of antifungal agents, pyrrolnitrin, and two derivatives of pseudane. In combination, these antibiotics gave the strain high activity against several fungal pathogens including *Pyricular oryza*, *R. solani* and *Verticillium dahliae* . Burkhead, *et al*., (1994) also reported that pyrrolnitrin was produced by *B. cepacia* isolate B37w which was very effective against the economically important potato pathogen fungus, *Fusarium sambucinum* . El-Banna and Winkelmann (1998) extracted pyrrolnitrin produced by *B. cepacia* NB-I, which exhibited a broad spectrum of antimicrobial

activity against 8 filamentous fungi (*Trichoderma pseudokonigii* was the primary test species), 2 yeasts, 1 Gram negative and 19 Gram positive bacteria.

Hwang *et al*. (2002) compared and quantified the production of pyrrolnitrin by four different strains of *B. cepacia* by TLC and HPLC, and showed that *B. cepacia* strain 5.5B and RR 21-2 produced large quantities of pyrrolnitrin . De Souza and Raaijmakers, (2003) extracted and identified pyrrolnitrin by TLC in several *B. cepacia* isolates J2535, LMG 1222 and ATCC 51671, that were suppressive against *Gaeumannomyces gramimis var. tritici* . Kadir, *et al*., (2008) extracted pyrrolnitrin and three other antifungal compounds produced by *B. cepacia* strain B23, which inhibited the growth of *Colletotrichum gloeosporioids* . Sultan, *et al*., (2008) isolated pyrrolnitrin and two novel oxidized derivatives from *B. cepacia* K73, which inhibited the growth of *R. solani* .

1.4.2.1.2 Bactobolin

Bactobolin (Figure 1.2) is a highly polar compound with strong antimicrobial and antitumor activity, and is structurally related to actinobolin produced by *Streptomyces griseoviridis* (Seyedsayamdost, et al., 2010). Bactobolin was first isolated from *Pseudomonas* BMG13-A7 (Kondo*, et al.*, 1979), and later isolated from the *Burkholderia* species, such as *B. thailandensis* E264 and *B. pseudomallei* (Seyedsayamdost*, et al.*, 2010).

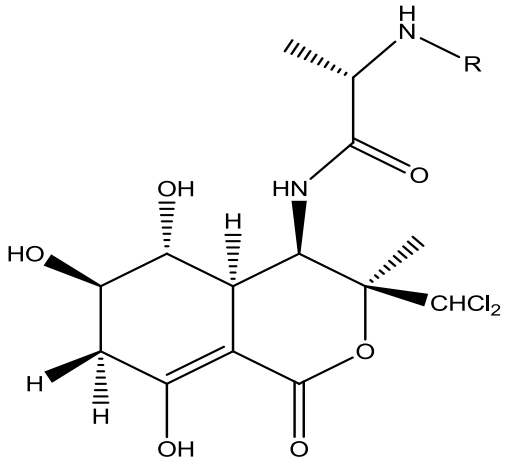


Figure1. 2 The structure of Bactobolin A

1.4.2.1.3 Quinolines

Quinolines (HMQ) (Figure 1.3) are strong antimicrobials that also act as iron chelators and signalling molecules; they are produced by many bacteria including *Pseudomonas aeruginosa* and several *Burkholderia* species (Vial*, et al.*, 2008). Moon, *et al*., (1996) isolated three quinolines metabolites (3-methyl-2-pentyl-4-quinolinone, 2-heptyl-3-methyl-4-quinolinone and 3-methyl-2-nonyl-4-quinolinone (HAQs and HMAQs)) from "*P. cepacia"* PCII. HMQs inhibited the growth of *Phytophthora capsici*, the causal agent of red pepper blight (Moon*, et al.*, 1996).

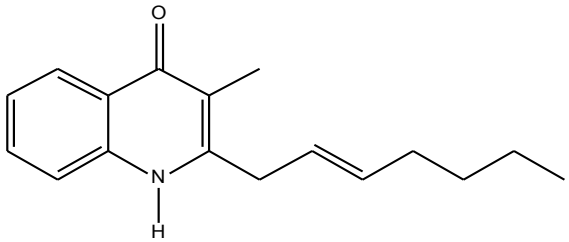


Figure1. 3 The structure of 4-hydroxyl-3- methyl-2-alkyl-quinoline

1.5 The production of *Burkholderia* antimicrobial agents

The production of antimicrobial agents, which are often secondary metabolites, is dependent on the primary metabolites that are produced by the catabolism of carbon, nitrogen and other important nutrients utilized by the microorganisms. These primary metabolites are considered to be precursors and suppliers of building blocks for secondary metabolism through different pathways. Multiple reports have demonstrated that the production of antibiotics occurs late in the stationary phase, as a result of exposure to extreme conditions, nutrient deficiency or feedback phenomena (Martin & Demain, 1980, Roitman*, et al.*, 1990, El-Banna & Winkelmann, 1998).

Various media have been used to grow bacteria and fungi in biological control studies. Microorganisms growing on different media can produce different metabolites and show different growth patterns (Jayaswal*, et al.*, 1990, Sfalanga*, et al.*, 1999, Hwang*, et al.*, 2002). The production and activity of antimicrobial agents fluctuates and is a very sensitive process that needs special conditions to actually have an effect on a target organism. These conditions are different and vary from organism to organism (Leisinger & Margraff, 1979, Duffy & Defago, 1999, Sfalanga*, et al.*, 1999, Raaijmakers*, et al.*, 2002, Bae*, et al.*, 2007, Li*, et al.*, 2007).

The production of secondary metabolites, which exhibit antimicrobial activity by Bcc isolates, is controlled by several factors such as media composition, pH and temperature (El-Banna & Winkelmann, 1998). Carbon, nitrogen and phosphate are the major requirements for the growth and metabolic activities of microorganisms. Carbon source greatly influences

microbial secondary metabolite production. The production of antimicrobial agents, such as pyrrolnitrin, by Bcc was affected by changing the source of carbon, with enhanced production occurring after growth on glycerol (Roitman*, et al.*, 1990, El-Banna & Winkelmann, 1998, Bae*, et al.*, 2007).

Nitrogen is one of the major requirements because it is needed for the synthesis of cell components and functional proteins. It is well-known that the different kinds of nitrogen source greatly influence microbial secondary metabolite production. Nitrogenous compounds may influence the biosynthesis of antibiotics directly at the level of secondary metabolism, either through their availability as substrates for antibiotic biosynthesis or through the modulation of biosynthesis and activities of the enzymes. The biosynthesis of many different types of antibiotics is also regulated by phosphate (Aharonowitz, 1980). Inorganic phosphate controls the synthesis of a large number of antibiotics that belong to different classes such as macrolides, tetracyclines and aminoglycosides (Martin & Demain, 1980, Martin, 2004). However, high concentrations of inorganic phosphate can repress the biosynthesis of antibiotics (Kishimoto*, et al.*, 1996). The pH of growth media also exerts an effect on the production of antimicrobial agents. The pH may change the ionizing state of important essential molecules such as proteins and enzymes. The production of antimicrobial agents is dependent on multiple enzyme catalysed reactions. Therefore, pH is one of the most important physical factors affecting antimicrobial production and activity. In general, pH ranging from 6.0 - 8.0 was optimal for antibiotic production by *Burkholderia* bacteria (Roitman*, et al.*, 1990, El-Banna & Winkelmann, 1998, Li*, et al.*, 2007).

1.6 Extraction, purification and identification of antimicrobial agents

Microbial secondary metabolites are chemical compounds with low molecular masses that are often formed during the stationary phase in response to the limitation changes in the growth conditions (Ruiz-Lozano & Bonfante, 2000). These metabolites may or may not exhibit antimicrobial activity. Many studies demonstrated various methods of extraction, characterization and identification of antimicrobial agents produced by Bcc and other *Burkholderia* bacteria.

1.6.1 The extraction of antimicrobial agents

After the detection of antimicrobial activity in a pure culture, the extraction of active compounds is the next important step if the structure and specific activity of the antimicrobial agents are to be characterized in detail. The development of suitable sample preparation techniques is needed to provide high quality, active and pure natural products in sufficient quantities. Several studies have used various methods of extraction and identification to characterize antimicrobial agents produced by *Burkholderia*. Antifungal *Burkholderia* compounds have been extracted from *Burkholderia* metabolites using extraction techniques such as maceration, followed by liquid partition in a wide range of different solvents with different polarities and concentrations. Solvents such as methanol (Moon*, et al.*, 1996, Kadir*, et al.*, 2008), acetone (Roitman*, et al.*, 1990, Burkhead*, et al.*, 1995, El-Banna & Winkelmann, 1998), chloroform (Homma*, et al.*, 1989) and a mixture of chloroform and methanol (Jiao*, et al.*, 1996) have been used to extract active *Burkholderia* metabolites.

Another successful method for the extraction of antibiotics from microbial cultures, which reduces the reliance on solvent extraction, is to use adsorption onto neutral resins such as Amberlite. For example, Amberlite XAD-16 has been shown to be a very efficient means to purify erythromycin from solution (Ribeiro & Ribeiro, 2003). These resin extraction protocols were recently applied by Mahenthiralingam, *et al*., (2011) to purify the polyketide enacyloxin from *B. ambifaria* AMMD, however, the wider exploration of utility of the resin adsorption methods in extraction of *Burkholderia* antibiotics remains to be carried out.

1.6.2 Purification of antimicrobial agents

Chromatography is a very commonly used technique in chemistry for separation, characterization and identification of compounds, firstly introduced by the Russian botanist Micharl Iswett. There are many types of chromatography with Thin Layer Chromatography (TLC) being one of the simplest, most rapid and economical of the procedures available. TLC separations take place in the thin sorbent layer such as silica gel, alumina or ion exchange resins which are supported on a glass, metal or plastic plate. Each extracted component is subjected to the same total separation time, but different migration distances occur based on the nature of the chemical components. These TLC chemical profiles can be visualized, depending on the nature of the compounds, in either normal day light, UV light (254 and 365 nm), or located using a stain procedure with iodine/other chemical spray reagents.

The degree of TLC fractionation depends on the nature of the chemical mixture under investigation, the type of adsorption layer and the power (polarity) of the mobile phase (Fritz

& Schenk, 1987). The retention (retardation) factor (R*f*) is commonly used to describe the specific chromatographic fractionations observed in a given TLC profile. The R*f* value is the ratio of the distance the solvent travels to the distance the compound travels, with the center of each spot taken as the point from which the measurement of the migration in relation to the solvent front is made. R*f* values and other characteristics such as the colour of metabolites make it possible to qualitatively study the nature of complex chemical mixtures; it is ideal as a simple method to examine microbial secondary metabolite mixtures. TLC can be further expanded to measure the bioactivity of secondary metabolites if, for example, the fractionated compounds are overlaid with a soft agar containing an indicator microorganism or cell suspension. A microbial bioassay was developed in this current project to study the diversity of antifungal agents present in *Burkholderia* secondary metabolites (chapter 4).

1.6.3 Identification of antimicrobial agents

A number of methods can be used to characterise the active antimicrobial compounds including liquid-liquid chromatography (LC), mass spectrometry (MS), infrared spectrometry (IR), ultra-violet (UV) absorption and nuclear magnetic resonance (NMR). A combination of LC-MS can be used to study multi-component mixtures and detect specific compounds within them. This method can be used to help identify known classes of compounds by mass when searching for novel bioactive compounds. Molecular weight and UV absorbance data of bioactive natural products in MS databases allow rapid identification of compounds. MS is also used in conjunction with NMR spectroscopy to determine the molecular formula of compounds. These data can then be compared to a database of compounds that have already

been identified. NMR is the ultimately method used to confirm the structural identity of a compound (Petr, 2008).

1.7 Antimicrobial activity testing

There are several different methods which can be use to investigate the efficacy of novel antimicrobials against susceptible microorganisms; these are often based on diffusion or dilution concepts. Diffusion methods include agar well diffusion assays, agar disk diffusion assays and bioautography assays, while dilution methods include agar dilution assay and broth dilution assay. The diffusion assay has been widely used to test biological extracts for antimicrobial activity. It is considered as a preliminary test used to determine the efficacy of biologically active compounds against susceptible microorganisms. The broth dilution assay can also provide useful information and define values, such as the Minimum Inhibitory Concentration (MIC), in terms of microbial growth suppression. MIC is the lowest concentration of an antimicrobial that can inhibit the visible growth of testing microorganism and is an important measure of the potency of new antimicrobial agents (Lorian, 2005). Bioautography assays were used for the first time by Goodal and Levi (1946) in their study of penicillin. Bioautography conducted TLC is a very helpful assay for testing active biological extracts for their antimicrobial effects (Rios*, et al.*, 1988).

1.8 The genetic basis of *Burkholderia* antifungal agents

The emergence of drug resistance among microorganisms is a major challenge of humankind. There is an urgent need to develop and discover new antibiotics to treat microbial infections.

In addition to using classical inhibition assays and chemical analysis to characterise antimicrobial agents, in the last few decades considerable research into the genetics of antibiotic production has been carried out. The first bacterial genome, published in 1995, was for *Haemophilus influenzae*, and we now have over 2000 complete bacterial genomes and multiple draft genomes available for analysis (www.genomesonline.org). Currently, the complete genomes of 20 strains of *Burkholderia* from different species and ecological categories can be examined online at the *Burkholderia* Genomes Database (BGD; www.burkholderia.com). Genomic information available on the publicly accessible databases, such as Genbank or BGD, can be investigated to increase our understanding of the genes responsible for biosynthesis of metabolites with antimicrobial activity.

One of the most powerful approaches used to identify the function of microbial-encoded genes is to introduce mutations into the bacterial genome and screen for mutants which have lost specific biological functions. Mutation of genes using transposons is a particularly useful approach to characterize gene function in bacteria (Steiniger-White, *et al.*, 2004). Transposition is a recombination process in which transposable elements, such as Mu, Tn3, Tn5 or Tn10, move from one site on a genome to a new site on the same genome, or move from a mobile element such as plasmid into a completely new genome. The movement of transposons in this way can cause genetic rearrangements such as deletions, inversions or duplication of DNA sequences, as well as inactivation of the genes within which they insert, and sometimes activation of genes adjacent to the site of insertion (Steiniger-White, *et al.*, 2004). Transposon mutants lacking the ability to produce antimicrobial agents have been used to investigate the role of antibiotics in the biocontrol of microorganisms (Fravel, 1988).

There are multiple bioinformatic tools available that can aid the identification of known secondary metabolite genes within genomes. In addition, such genome mining may also lead to the discovery of novel antibiotics within *Burkholderia* genomes such as *B. ambifaria* AMMD. Artemis is a genome sequence visualisation and analysis tool developed by Rutherford *et al*., (2000) (http://www.sanger.ac.uk/resources/software/artemis/). The software allows, on one screen, the viewing of the sequence of an entire genome in a graphical format; additional tools such as the Artemis Comparison Tool (ACT) also enable the display of pair-wise comparisons between two or more genomes (Carver*, et al.*, 2005). Genome annotation is the identification of putative functional roles for genes encoded within a DNA sequence (Lukashin & Borodovsky, 1998). The BGD (www.burkholderia.com) provides similar features to Artemis but allows *Burkholderia* genomes to be viewed and compared over the web. Hence, this tool can allow the comparison of *B. ambifaria* AMMD to all the additional *Burkholderia* genomes available. It is a very powerful tool with which to identify the presence of orthologous genes in *Burkholderia* genomes.

Genomics is a fast-growing field and provides a major resource for screening and identification of gene(s) and pathways that might have a potential role in solving many worldwide biological problems. Bioinformatics is a good tool for identification of known secondary metabolite gene homologs. This may lead to the discovery of novel and existing antifungal antibiotics.

Aims

Secondary metabolites produced by biocontrol microorganisms, especially from Bcc bacteria, show considerable potential as antimicrobial agents. **The overall aim of the current work was to screen, isolate, characterise and identify *Burkholderia* antifungal agents, investigating their natural diversity and activity on both ascomycete and basidiomycete fungi.** A broad range of research covering the following objectives was carried out:

Objective 1: Screening of *Burkholderia* isolates for the antifungal production.

A large collection of accurately identified *Burkholderia* isolates held by Mahenthiralingam group at Cardiff University was screened for the production of antifungal agents. A general hypothesis for this work was "all *B. cepacia* complex bacteria are capable of producing antifungal agents" **(Chapter 3)**. The objectives were to:

- Evaluate a range of growth media for their ability to support bacterial growth, and allow the production of antimicrobial metabolites.
- Systematically screen a collection of *Burkholderia cepacia* complex species for the production of antifungal metabolites.
- Examine the secretion of antifungal metabolites by *Burkholderia* species outside the *B. cepacia* complex and compare this with a range of non-*Burkholderia* species.
- Develop an overlay-inhibition assay suitable for screening antifungal activity against filamentous basidiomycetes.

Objective 2: Analysis of the chemistry of antifungal substances secreted by *Burkholderia*.

A range of different chemical methods were used to purify and explore the chemistry of *Burkholderia* antibiotics. The general hypotheses for this chapter were: "*Burkholderia* bacteria produce more than one antimicrobial compound and TLC-bioautography can be used to examine *Burkholderia* antimicrobial diversity." **(Chapter 4)** The objectives were to:

- Investigate the nature of *Burkholderia* secreted products by development of rapid extraction methods.
- Optimize conditions of thin layer chromatography to enhance fractionation and visualization of *Burkholderia* active metabolites.
- Optimize the bioautography assay to detect active agents in each extract.
- Study the diversity of antimicrobial agents within *Burkholderia*.
- Purify, isolate and identify *Burkholderia* active metabolites using TLC, liquid chromatography and mass spectroscopy.
- Determine the minimal inhibitory concentrations of crude and semi-purified *Burkholderia* active metabolites.

Objective 3: Optimization of antibiotic production in antifungal *Burkholderia* isolates.

The components of synthetic media and different factors such as pH, temperature and incubation period were investigated, with enhancement of antimicrobial production by *Burkholderia* as the overall aim. A general hypothesis was ''chemical and physical factors can

influence anti-candidal production and activity of *Burkholderia*'', **(Chapter 5)** and the specific objectives were to:

- Examine the effects of six different factors: carbon, nitrogen, phosphate, pH, incubation time and temperature on the activity of anti-candidal agents of *Burkholderia*.
- Create an optimal medium to increase anti-candidal activity of *Burkholderia* metabolites.

Objective 4: The natural diversity of antifungal producing bacteria associated with different rhizosphere environments.

This was investigated with a culture-dependent approach using selective media to enrich for antibiotic producing bacteria and molecular methods to identify these bacteria. A general hypothesis for this research was "a rhizosphere bacterial isolates are capable of producing one or more antifungal agents", **(Chapter 6)** and the specific objectives were to:

- Isolate antifungal producing bacteria from different rhizosphere environments.
- Identify the antifungal producing bacterial species using 16SrRNA.
- Extract antifungal agents synthesised by different isolates.
- Study the presence and diversity of antifungal agents in antifungal producing bacteria using a developed TLC-bioautography assay.

Objective 5: Identification of the genetic basis for the secretion of antifungal agents by *Burkholderia*.

This research examined the genetics behind the production of anti-fungal agents by *Burkholderia* using molecular genetics techniques such as transposon mutagenesis and genome sequence analysis. A general hypothesis was ‘‘mutation and bioinformatics analysis can predict the genes involved in antimicrobial secretion by *B. ambifaria* AMMD’’ **(Chapter 7).** The specific objectives were to:

- Identify the genes responsible for the production of antimicrobial agents in *B. ambifaria* AMMD by transposon mutagenesis.
- Detection the presence or absence of anti-candidal agents in isolated transposon mutants using antimicrobial extraction followed by TLC-bioautography assay.
- Use bioinformative analysis to characterise orthologs of *B. ambifaria* secondary metabolites genes (upregulated during growth on glycerol) in other *Burkholderia* genomes.
- Characterise the kinetics and physiology of *Burkholderia* metabolite secretion over time.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

Materials and Methods

2.1 Microbial growth media

The media used in current study (Table 2.1) were basal salts minimal growth media (BSM) with glycerol as a carbon source (BSM-G), Tryptone Soya broth/ agar (TSA/TSB), Potato dextrose agar (PDA), Sabouraud agar (SA), 2% Malt agar (MA), Muller Hinton agar (MHA); Muller Hinton broth (MHB), Isosensitest agar (ISOA), soft Isosensitest agar (SISOA, see below), Casein meat agar (CAM) and Peptone Lactose agar (PLA). All premixed media, individual nutrient components and agar were obtained from Oxoid UK unless indicated otherwise. BSM-G was composed of (gl^{-1}): $K_2HPO_4.3H_2O$, 4.25; $NaH_2PO_4.H_2O$, 1.0; NH_4Cl, 2; nitrilotri acetic acid (C_6H_9NO), 0.1; $MgSO_4.7H_2O$; 0.2, $FeSO_4.7H_2O$, 0.012; $MnSO_4.H_2O$, 0.003; $ZnSO_4.7H_2O$, 0.003; $CoSO_4.7H_2O$, 0.001; 0.05% Cas amino acids; 0.05% yeast extract and 0.4% glycerol as a carbon source (m/m). To make solid BSM-G media, 15 gl^{-1} of purified agar was added. The carbon source in BSM was altered as described (Section 2.7.1) for the experiments to examine the induction of antimicrobial biosynthesis. 2% Malt agar medium (MA) consisted of (gl^{-1}): malt, 20 and purified agar, 15. Soft Isosensitest agar medium (SISOA) was prepared by addition of purified agar 10 gl^{-1} (1%), to standard Isosensitest broth prepared to the manufacturer's recipe. Casein meat agar (CAM) was prepared by combining (gl^{-1}) pancreatic digest of casein, 5.0; meat extract, 5.0; and purified agar 15. Peptone Lactose agar (PLA) was prepared by mixing (gl^{-1}) Neo Peptone, 10; Lactose, 10 and purified agar 15. All media were prepared with sterile polished water and sterilised by autoclaving at 121°C for 15 minutes.

Table 2.1 Formulation of growth media used in this study.

Medium (supplies)	Abbreviation	Carbon source	Protein source	pH (25°C)
Basal salts media	BSM	Glycerol	Amino acids	7.0± 0.2
Tryptone Soya broth/agar (Oxoid)	TSA &TSB	Unidentified	Peptone	7.0± 0.2
Potato dextrose agar (Oxoid)	PDA	Starch & glucose	Unknown	5.6± 0.2
Sabouraud broth/agar (Oxoid)	SB & SA	Dextrose	Peptones	5.6± 0.2
2% Malt agar	MA	Starch	Unknown	5.6± 0.2
Muller Hinton agar/ broth (Oxoid)	MHA	Starch	Beef & Casein	7.0± 0.2
Isosensitest agar / broth (Oxoid)	ISO& ISOB	Starch & glucose	Peptones, Casein & amino acids	7.0± 0.2
Casein meat agar	CAM	Unidentified	Casein & meat	5.6± 0.2
Peptone Lactose agar	PLA	Lactose	Peptone	6.0± 0.2

2.2 Microorganisms storage and maintenance

2.2.1 Bacteria

A total of 421 bacterial isolates (397 *Burkholderia* isolates; Table 2.2) from a variety of sources including cystic fibrosis infection (designated as CF), various clinical infections (designated as Non-CF) and the environment (designated as ENV; these included plant pathogenic, soil, and isolates from various environmental sources including industrial contamination) were obtained from the Cardiff University collection (Mahenthiralingam, *et al.,* 2008). Reference and type strains were obtained from the Belgian Co-Ordinated Collections of Microorganisms (LMG BCCM) (http://bccm.belspo.be/about/lmg.php). Control strains included: *Staphylococcus aureus* (NCTC12981), *B. multivorans* (ATCC17616; BCC0011), *E. coli* (NCTC12241), *Pseudomonas aeruginosa* (NCTC12903), and other multidrug resistance bacteria *S. aureus* Eagles, *S. aureus* EMRSA15*, S. aureus* MRSA Mu50, *B. cenocepacia* (LMG16656) [BCC0665]*, P. putida* (LMG 2257^T) *P. fluorescens* (LMG 1794^T)*, Stenotrophomonas maltophilia* (LMG 958^T)*, Acinetobacter baumannii* (OXA-23) and *Enterococcus faecalis* (ATCC 51299).

Bacterial isolates (a suspension of fresh plate or liquid growth) were stored at –80ºC in vials containing TSB medium with 8% (vol/vol) di-methyl sulphoxide (DSMO). To revive isolates, vials were removed from the -80ºC freezer, the bacterial stock surface swabbed with a sterile cotton swab while deep frozen, immediately plated onto a fresh TSA plate and streaked out to single colonies by a sterile loop. Plates were incubated at 30ºC or 37ºC as appropriate for the

Table 2.2 List of *Burkholderia* isolates and other bacteria used in this study.

***Burkholderia* species**	**No. of tested isolates**	**Habitat**		
		CF [a]	**Non-CF [b]**	**ENV [c]**
***B. cepacia* complex (339 isolates)**				
B. ambifaria	101	6 (1 Control [d])	-	95 (1 Control)
B. cenocepacia	61	27 (8 Control)	9 (4 Control)	21
B. cepacia	30	11 (1 Control)	4 (1 Control)	15 (4 Control)
B. multivorans	27	14 (3 Control)	4 (1 Control)	6 (1 Control)
B. vietnamiensis	18	5 (1 Control)	3	10 (3 Control)
B. stabilis	18	5	5	6
B. pyrrocinia	16	2	-	14 (2 Control)
B. anthina	14	5 (2 Control)	-	9 (4 Control)
B. lata	11	1	-	9 (1 Control)
Bcc novel groups Bcc 6	10	1	-	9
B. dolosa	8	8	-	-
B. contaminans	6	4 (1 Control)	-	2
Bcc novel groups Bcc 5	5	1	1	3
B. arboris	3	1 (1 Control)	-	2
Bcc novel groups Bcc 4	3	1 (1 Control)	-	2
B. lateens	2	2 (1 Control)	-	-
B. seminalis	2	1	-	1
Bcc novel groups Bcc Kc	2	-	-	2 (1 Control)
B. diffusa	1	-	-	1 (1 Control)
Bcc novel groups Bcc 8	1	1	-	-
***Burkholderia* species not Bcc (58 isolates)**				
B. gladioli	12	9	-	3
B. fungorum	3	3	-	-
B. terricola	1	-	-	1 (1 Control)
B. kururiensis	1	-	-	1(1 Control)
B. mimosarum	1	-	-	1 (1 Control)
B. oklahomensis LMG23618	1	-	-	1 (1 Control)
B. phenazinium	1	-	-	1 (1 Control)
B. phenoliruptrix	1	-	-	1 (1 Control)
B. phymatum	1	-	-	1 (1 Control)
B. phytofirmans	1	-	-	1 (1 Control)
B. plantarii	1	-	-	1 (1 Control)
B. sacchari	1	-	-	1 (1 Control)
B. xenovorans	1	-	-	1 (1 Control)
B. glathei	1	-	-	1 (1 Control)
B. glumae	1	-	-	1 (1 Control)
B. graminis	1	-	-	1 (1 Control)
B. hospital	1	-	-	1 (1 Control)

Table 2 Continued

B. andropogonis	1	-	-	1 (1 Control)
B. caledonica	1	-	-	1 (1 Control)
B. caryophlli	1	-	-	1 (1 Control)
B. phenaziniumi	1	-	-	1 (1 Control)
B. tropica	1	-	-	1 (1 Control)
B. tuberum	1	-	-	1 (1 Control)
B. caribensis	1	-	-	1 (1 Control)
Unidentified not Bcc	18	14	-	4

Other G^+ and G^- bacteria (24)	**Comments**
S. aureus NCTC12981	Antibiotic reference strain
S. aureus Eagles	Multidrug resistance strain
S. aureus EMRSA15	Multidrug resistance strain
S. aureus MRSA Mu50	Multidrug resistance strain
B. multivorans ATCC17210	
B. cenocepacia LMG16656	Multidrug resistance strain
P. aeruginosa NCTC12903	Antibiotic reference strain
P. fluorescens	Multidrug resistance strain
P. putida LMG 2257	
P. stutzeri	
Pand. Apista	
Pand. pnomenusa 2 isolates	
R. mannitolytica	
E. coli NCTC12241	Antibiotic reference strain
A. baumannii	Multidrug resistance strain
A. xylosoxidans	
E. faecalis	
S. maltophilia	

[a] : Isolates from the natural environment.
[b] : Isolates from cystic fibroses patient.
[c] : Clinical isolates not from cystic fibroses patient.
[d] : Fully identified isolates from the LMG BCCM (http://bccm.belspo.be/about/lmg.php).
G^+: Gram-positive bacteria.
G^-: Gram-negative bacteria.
Control: Reference and type strains (LMG, ATCC or NCTC strains)

microorganisms until suitable confluent growth had been obtained. For inoculation of liquid growth media and soft-agar overlays an inoculum suspension of fresh growth for each strain was prepared as follows: A few colonies of a freshly grown bacteria were inoculated aseptically into with 3 ml of a TSB medium (contained with a 15 ml tube) and incubated horizontally with shaking (New Brunswick; at 150 rpm) at 37ºC for 6 to 8 h. The liquid culture was then diluted to an optical density (OD) of 1 at 600 nm (corresponding to an approximate viable count of $1x10^8$ colony forming units per ml; cfu ml^{-1}). Different amounts of these standard suspensions were then used to inoculate starter cultures or soft-agars to reach the desired viable cell density appropriate for the assays described below.

2.2.2 Fungi

Candida albicans Sc 5314 (Table 2.3) was used as a reference strain to rapidly test the activity of antifungal agents produced by *Burkholderia* isolates. Pure cultures were maintained in cryogenic storage vials containing 1.5 ml TSB medium supplemented with 8% DMSO as described above. A *C. albicans* inoculum culture was prepared as follows: one colony of freshly grown bacterial cells was mixed with 9 ml of a sterilized TSB medium (contained with a 50 ml tube) and incubated horizontally with shaking (150 rpm) at 37ºC for 24 h; the culture was then diluted to an optical density of 0.35 at 530 nm using spectrophotometer, 8% DMSO added and stored as 1 ml aliquots which could be thawed and used as standard inocula corresponding to a viability of approximately 4.7×10^6 cfu ml^{-1}.

Table 2.3 Wood decay basidiomycetes and ascomycete reference isolates used as an indication of *Burkholderia* antifungal activity.

Fungal isolates	Isolate code	Characteristics
Basidiomycete isolates		
Hypholoma fasciculare	HFGTWV2	Late coloniser, forms mycelial cords
Hypholoma fasciculare	HFDD2	Late coloniser, forms mycelial cords
Resinicium bicolor	Rbm6A	Late coloniser, forms mycelial cords
Resinicium bicolor	Rb1	Late coloniser, forms mycelial cords
Phanerochaete velutina	PV29	Late coloniser, forms mycelial cords
Phallus impudicus	PIJHYA	Late coloniser, forms mycelial cords
Stereum gausapatum	Sa1	Primary coloniser
Bjerkandera adusta	BK1	Secondary coloniser
Trametes versicolor	D2	Secondary coloniser
Heterobasidion annosum	SP313	Tree root pathogen and butt rot
Ascomycete isolate		
Candida albicans	Sc 5314	Yeast, control strain

Ten representative species of cord-forming wood decay basidiomycetes fungi (Table 2.3) were provided by Prof. L. Boddy, Bioscience, Cardiff University. These fungi were used in this study to detect the activity of antifungal agents produced by *Burkholderia* isolates. A fungal inoculum was aseptically transferred to a tube containing slant of 2% MA medium and allowed to grow for 5 d at 20ºC, the stock cultures were then stored at 4ºC. For revival, the slant culture was removed from the 4ºC refrigerator and a small portion of fungal mycelium transferred with a sterile needle to a fresh 2% MA plate and incubated at 20ºC.

2.3 Sampling and isolation of Bcc and other bacteria from natural environments

Soil samples were collected from mixed deciduous woodland in the Coed Beddick**,** Tintern**,** U.K (coordinates 51.710690, -2.681351) in August 2009. Maize rhizosphere and soil samples were collected with permission from fields around Bridgend, Wales, UK (coordinates 51.513837,-3.645841) in July 2010. To remove unwanted materials, such as wood litter and big stones, samples were sieved (1.50 and 0.50 mm).

Bacteria were cultivated from soil samples by adding 0.1 g of sieved soil to 700 µl of BSM-G media supplemented with cycloheximide (100 mg l^{-1}), and polymyxin (400 µg l^{-1}) (designated as BSM-CP), and gently homogenised by hand with a plunger that fitted the 1.5 ml microtube in which the sample was placed. After heavy debris had been allowed to settle out, 200 µl of soil homogenate was added to 2 ml of BSM-CP in a 14 ml snap top tube and incubated horizontally (shaking at 150 rpm) at 30ºC for 24h, then 200 µl of each culture were transferred to a sterile micro-tube containing 16 µl DMSO (8%) and stored at -80ºC until use. To isolate bacteria from the maize rhizosphere, maize roots were

aseptically removed from each plant by cutting about 0.5 to 1 cm portion with a sterilized blade and placing them in a microtube.

Each root section was then washed twice by gentle vortex mixing with sterilized distilled water (1 ml) to remove any loosely adhering soil particles. The washed root sections were placed in 700 µl of BSM-CP and gently homogenised with a plunger. After allowing the heavy debris to settle out, 200 µl of the root homogenate was added to 2 ml of BSM-CP contained in a 14 ml snap top tube and incubated horizontally with shaking (150 rpm) at 30ºC for 24 h. After growth, 200 µl of each culture were transferred to a sterile micro-tube, DMSO added to 8%, and stored at -80ºC until use.

The soil and root homogenate cultures were screened for antimicrobial phenotypes against a wide range of microorganisms as follows. BSM-G plates were inoculated in the center with 5 µl of each culture and incubated at 30ºC for 3 d. The microbial growth was killed by exposing the plates to chloroform vapour for 2.5 min by placing each plate, agar face down on a wire mesh approximately 0.5 cm from chloroform within in a Pyrex dish. After chloroform vapour killing, the plates were transferred to a laminar flow cabinet for 10 minutes to remove residual chloroform by evaporation. Then, plates were overlaid with molten SISOA (45^{o}C) seeded with approximately 1 x 10^{6} cfu ml^{-1} with a specific antimicrobial susceptibility testing micro-organism (*C. albicans, B. multivorans* or *S. aureus*). 2, 3, 5-triphenyltetrazolium chloride (TTC) was added to a final concentration of 0.02% to the overlay agar as a metabolic stain to detect growth of the test organisms and aid visualisation of antibiotic inhibition zones. Soil or root homogenate cultures which showed antimicrobial activity in the above assays were plated to single colonies to enable

purification of each antimicrobial producing microorganism. Single colonies were then retested for antimicrobial activity using the overlay assay; pure positive isolates were identified using 16S rRNA gene sequence analysis (see section 2.8.2) and stored at -80ºC until use.

2.4 Identification of isolated bacteria

2.4.1 Isolation of genomic DNA

Overnight cultures of *Burkholderia* and other bacteria used in the study were grown in TSB (3 ml cultures) to stationary phase (18 to 48 h dependent on the strain). Bacterial cells were harvested by centrifugation and then re-suspended in 100 µl TE buffer (10 mM Tris-Cl pH 8; 10 mM EDTA, pH 8). The suspension was added to a 2 ml screw-cap micro-centrifuge tube containing approximately 500 µl of 0.1 mm zirconium beads (Biospec products inc., Bartlesville) and 500 µl lysis buffer (50 mM Tris –Cl, pH 8; 70 mM EDTA, pH 8; 1% sodium dodecyl sulphate; 0.5 mg ml^{-1} pronase). Bacterial cell lysis was achieved by a 10 sec pulse on a bead-beater machine (Biospec Products; Bartlesville, Okla.) and the lysate then incubated for 1 h at 37ºC to allow protein digestion. The lysate was centrifuged briefly to reduce bubbles, 200 µl of saturated ammonium was added to precipitate protein matter, and the lysate was pulsed for 5 sec on the bead-beater machine. Chloroform (600 µl) was added to the lysate and mixed by a 5 sec pulse on the bead- beater, a high speed centrifugation was then used to remove proteins and polysaccharides. Approximately 400 µl of the cleaned lysate was transferred to a sterile HI-YIELD 1.5 ml microtube containing 1 ml of 100% ice cold ethanol. After mixing, the tube was centrifuged for 7 min to collect the precipitated genomic DNA. The DNA pellet was washed with 500 µl of 70% ethanol and subjected to an additional centrifugation step to re-pellet the DNA. After removal of

all the ethanol the DNA pellet was dried under vacuum for 10 min and dissolved in TE-low EDTA buffer (10 mM Tris-Cl, pH 8; 0.1 mM EDTA, pH8) containing RNase A at 0.5 µg ml ml^{-1}. After incubation at 37ºC for 1 h to degrade any RNA remaining, the genomic DNA was stored frozen at – 20ºC until use.

To examine the quality and roughly estimate the quantity of the isolated DNA, 10 µl was loaded onto a 1.25% agarose gel (Bioline Ltd, London, UK) made with Tris-Borate-EDTA (TBE) buffer. The molten agarose was supplemented with Safe View dye (NBS Biologicals, Huntingdon; 2.5 µl Save View per 100 µl agarose gel) to enable the DNA to be subsequently vizualised under UV illumination. A DNA size ladder (10 µl of 1 kb molecular ladder; Newengland Biolabs UK.) was also loaded and then, electrophoresis performed at 100 volts for about 30 min in 1 x TBE buffer containing Safe View dye (2.5 µl per 100 ml TBE). Then the agarose gel was viewed under UV light at 245 nm and images were taken using a gel documentation camera (Syngene Gene Snap, Cambridge UK).

2.4.2 Bacterial 16S rRNA gene amplification and sequence analysis

Pure bacterial cultures isolated from maize or forest soils were identified using 16S rRNA gene sequence analysis. Primers 27F (5'- AGA GTT TGA TC TGG CTC AG -3') and 1492R (5'- GGT TAC CTT GTT ACG ACT T -3') were used to amplify 16S rRNA gene as follows: The total volume of each reaction was 25 µl and contained: 10 to 40 ng of genomic DNA (2 µl of the concentrated stock solutions that had been diluted tenfold), 13.8 µl sterile nuclease free H_2O, 2.5 µl 10 x Coralload buffer, 5 µl 5 x Q-Solution, 0.5 µl 10 mM dNTPs, 0.5 µl of each primer (4 pmol μl^{-1} final concentration) and 0.2 µl (1 unit) Taq

DNA polymerase (Qiagen Ltd., Carwley, UK). PCR was carried out in a DNA Engine Dyad Thermal cycler gradient block (MJ Research, Boston, MA) for 2.5 h with the thermal cycle as described in Table 2.4. PCR products were evaluated by agarose gel electrophoresis as described above except that 1.5% agarose was used. Pure 16S rRNA gene products were sequenced using primers 27F and 1492R with the Applied Biosystems Big Dye Terminator ready reaction mix version 3.1 followed by analysis on an Applied Biosystems ABI-Prism 3100 automated sequencer. The identity of each isolate was determined by submitting the resultant 16S rRNA gene sequence to the Basic Local Alignment Search Tool (BLAST) at the National Centre for Biotechnological Information (NCBI; http://www.ncbi.nlm .nih.gov/) and searching for the closest matches.

Table 2.4 PCR cycles for 16S rRNA gene amplification.

Step	**Temp. Cº**	**Time**	**Nº cycles**
1. Initial DNA denaturation	95	00.02.00	1
2. DNA denaturation	94	00.00.30	35
3. Primer annealing	52	00.00.30	
4. Primer extension	72	00.01.30	
5. Indefinite hold	72	00.05.00	1

2.5 Antimicrobial testing

2.5.1 Screening of antimicrobial activity using agar overlay assay

To test the ability of *Burkholderia* isolates to produce antimicrobial agents, a 3 µl drop of each *Burkholderia* frozen inoculum culture (approximately 1 x 10^8 cfu ml^{-1}) was spotted onto the centre of two different media (SA or BSM-G) and incubated at 30ºC for 3 d. The bacterial growth was chloroform vapour killed as described above (section 2.3) and an overlay agar assay was used as a rapid technique to detect which *Burkholderia* isolates were antimicrobial producers (Figure 2.1). To prepare overlay media, 100 ml of molten SISOA (45ºC) was aseptically seeded with the susceptibility testing organisms *C. albicans* (500 µl of stock to give approximately 1 x 10^6 cfu ml^{-1} of SISOA), or *S. aureus* (500 µl of stock to give approximately 1 x 10^6 cfu ml^{-1} of SISOA), or *B. multivorans* (100 µl of stock to give approximately 2 x 10^5 cfu ml^{-1} of SISOA). The growth indicator 2,3,5-triphenyltetrazolium chloride was also added to the SISOA to a final concentration of 0.02%, to aid visualisation of any resultant zones of clearing. Approximately 15 ml of the overlay culture was poured onto the surface of each *Burkholderia* colony. After allowing the soft agar to set at room temperature, the plates were incubated at 37ºC for 18 h. The antimicrobial activity was revealed by the presence of a zone of clearing, where the susceptibility testing microorganism failed to grow. The diameter of the zone of inhibition was measured (mm) and all assays completed in triplicate to allow estimation of the mean and standard error of the mean (SEM).

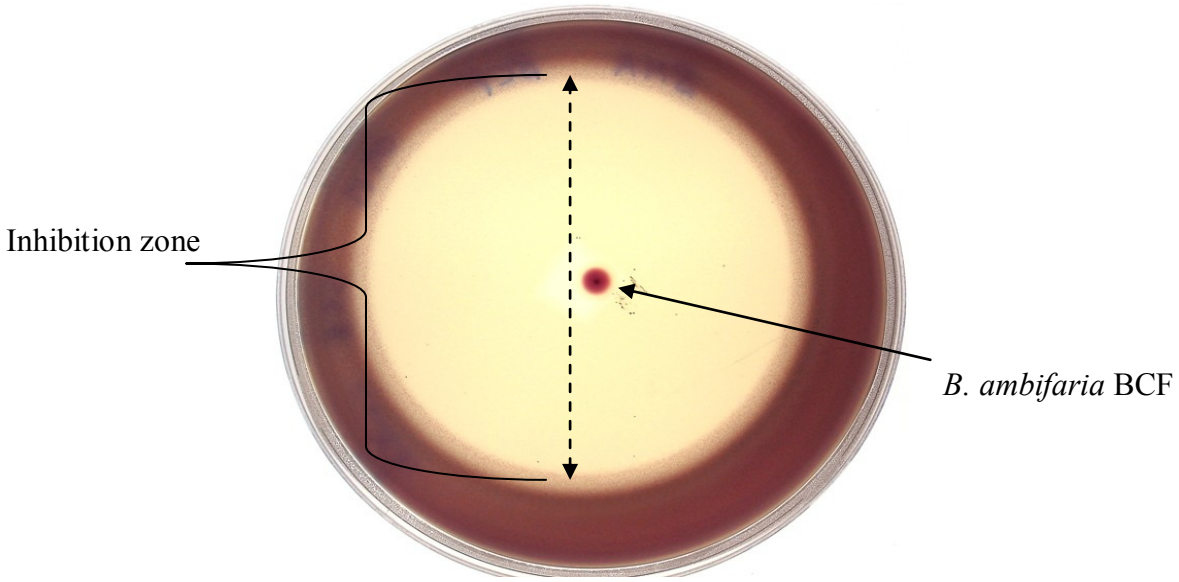


Figure 2.1 *C. albicans* overlay set-up to detect anti-candidal agents produced by *B. ambifaria* BCF.

Bacterial suspensions was spotted onto the center of BSM-G plate and incubated at 30°C for 3 days. The *Burkholderia* strain was killed by exposing to chloroform vapour then the plate was overlaid with Soft Isosensitest agar seeded with *C. albicans*. After incubation at 37°C for 24 h, the anti-candidal activity of strain BCF is clearly visible as a clear zone on the red background of Candida cells which have grown and reduced the tetrazolium chloride metabolic indicator.

2.5.2 Screening of anti-basidiomycete agents

To test the ability of *Burkholderia* isolates to produce anti-basidiomycetes agents, two different methods were used: the contact antagonism assay and a developed basidiomycete overlay assay.

2.5.2.1 Contact antagonism assay

The agar contact antagonism assay was used to detect the antagonistic ability of *Burkholderia* isolates against the model cord-forming wood decay fungus *Bjerkandera adusta* BK1 (BK1). A 3 µl drop of each *Burkholderia* inoculum (see section 2.5.1) was spotted onto one of four points on the SA plates as shown in Figure 2.2. The plates were incubated at 30ºC for 48 h to allow secretion of secondary metabolites by *Burkholderia*, then the plates were inoculated with a 5 mm agar plug of BK1 grown for 7 d on malt agar plates. Control plates of BK1 were also prepared. The plates were incubated for 7 d at 25ºC to allow interaction between BK1 and *Burkholderia* metabolites. The results were scored by comparison to BK1 control plates, noting whether there had been the following *Burkholderia* effect on growth of the fungal hyphae: no inhibition (-), weak inhibition (+, 0.1 cm to 0.5 cm antagonism of BK1 growth), medium inhibition (++, 0.5 cm to 1 cm antagonism of BK1 growth) or strong inhibition (+++, 1 cm to 2 cm antagonism of BK1 growth).

2.5.2.2 Basidiomycete agar overlay assay

Due to the filamentous nature of fungi it is difficult to obtain separated cells which can be used to evenly inoculate basidiomycetes onto media; the following methods involving

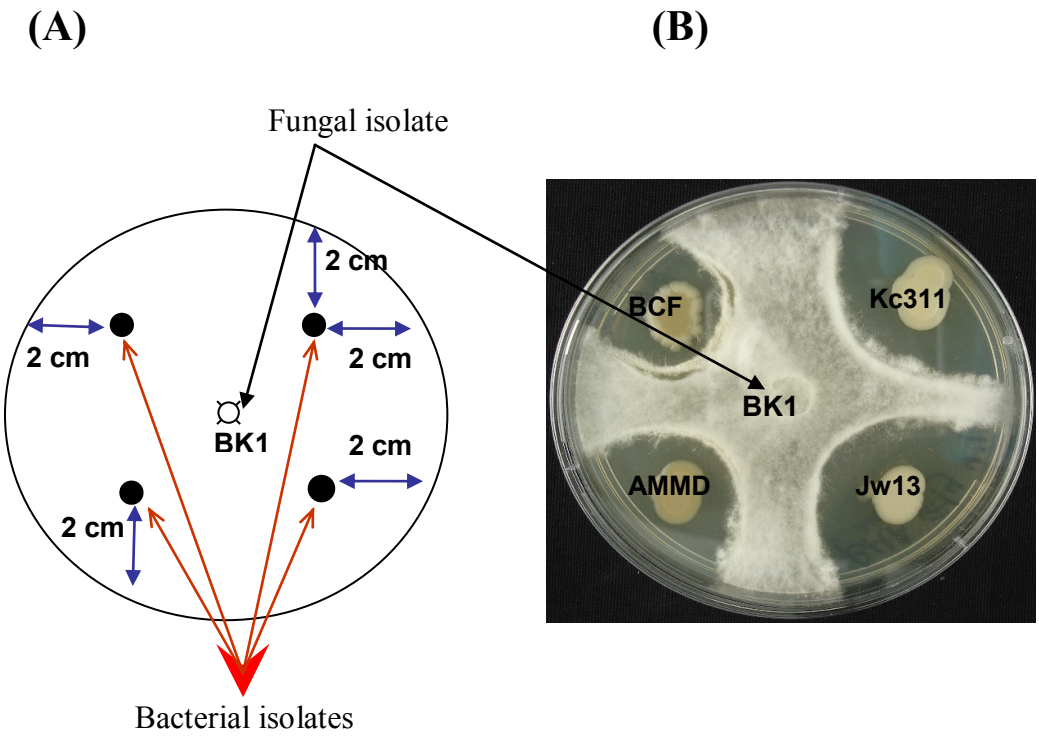


Figure 2.2 Interaction set-up of *Burkholderia* isolates and *Bjerkandera adusta* BK1 using contact antagonism assay.

(A) *Burkholderia* isolates were inoculated, 2 cm from the edge of the SA plate. After incubation at 30°C for 2 days, the plate was centrally inoculated with a 5 mm agar plug of *B. adusta* BK1 and incubated for 7 d at 25°C. The results were measured by comparison to a B. adusta control plate without any *Burkholderia*. (B) The resulting anti-*B. adusta* BK1activity is shown for *B. ambifaria* AMMD, BCF, Kc311 and *B. vietnamiensis* Jw13.

mechanical shearing of fungal mycelia to produce an even viable suspension for inoculation into an overlay agar was developed. Wood decay basidiomycetes (Table 2.2) were grown by inoculating 5 mm agar plugs for 7 d at 25°C on 2% MA plates. Superficial growth of fungal mycelia of each species was collected from the agar surface using a sterile blade. Approximately, 250 mg of each mycelium scraped and homogenized in a 2 ml tube, containing 1 ml of sterilized 2% MB and 500 mg of 0.25 mm sterilised zirconium beads (Biospec products inc., Bartlesville), using bead-beater machine for 30 sec at 25 rpm. Aliquots of this homogenised mycelial inoculum were kept at -20ºC until use. Fungal inocula were revived by re-culturing fragmented mycelial suspensions in 25 ml of 2% MB in 250 ml conical flask, and incubating at 25°C for 2 d at 150 rpm. Then 3 ml of fragmented mycelial suspension was mixed with 25 ml of 2% MA and overlaid on the top *Burkholderia* antibiotic production screen plates as described above (section 2.5.1); plates were then incubated at 25°C for 4 days to allow basidiomycete growth. The anti-basidiomycete activity of *Burkholderia* isolates was determined by measuring the diameter (mm) of the mycelial clearing zone.

2.5.3 Determination of antimicrobial activity using broth microdilution method

To determine the minimal inhibitory concentrations (MIC) of crude and semi-purified *Burkholderia* antimicrobial extracts the following was carried out (Sass, *et al*., 2011). The antimicrobial extracts were maintained in methanol, however, to avoid issues with the inhibitory effect of the methanol solvent, the extracts were dried using a rotary film evaporator, the dry weight of the material was determined and then dissolved in sterilized Muller Hinton broth (MHB). A 96-well plastic micro-plate (Sterilin, UK) was then used for the microdilution assay as follows. The total volume of culture in each well was 200 µl.

Serial dilutions of the antimicrobial extracts were performed in the test set of wells by mixing of 50 µl of MHB with 50 µl of the antimicrobial in MHB; control wells without antibiotic just contained 100 µl of MHB. The susceptibility testing microorganisms (100 µl in MHB; diluted from a fresh overnight culture that was standardized by optical density to 1.0 at 600 nm) were then added to each well to give a final inoculum of 1 x 10^6 cfu ml^{-1}. Then, 96-well plates were incubated with shaking (150 rpm) at 37ºC for 24 h. Microbial growth was quantified by measuring optical density at 600 nm using an automatic plate reader. The MIC value was identified as the lowest concentration of the antimicrobial extract which reduced the optical density to 80% of that of the control cultures; all MIC values were confirmed using triplicate wells.

2.6 Selection of suitable media for both growth and antifungal production

The growth of ten wood decay fungi (Table 2.3) was examined on seven different selected media (SA, PDA, MA, BSM-G, TSA, ISOA and MHA; Table 2.1). Each agar plate was inoculated at the centre by placing a 5 mm fungal colonized agar plug (from a 7 day culture grown on 2% MA) upside down in the centre of the plate. The plates were incubated at 25ºC and the radial extension (mm) was measured at 3, 5, 7, 9 and 11 d, extension rate was determined by linear regression.

Four Bcc isolates, *B. ambifaria* AMMD, BCF & Kc311 and *B. vietnamiensis* Jw13 known to produce antimicrobial agents were also tested for production of anti-candidal agents on the same media used to evaluate optimal growth of the wood decay fungi (SA, PDA, MA, BSM-G, CMA and MHA; Table 2.1). An antimicrobial production and overlay assay was

performed on each growth medium essentially as described in section 2.5.1. Briefly, a 3 µl drop of each isolate inoculum was spotted onto the centre or laterals of each agar plate and incubated at 30ºC for 3 d, bacteria were killed by exposure of the plates to chloroform vapour. After chloroform killing followed by aeration of the plates in a laminar flow cabinet, each plate was overlaid with either *C. albicans*, *S. aureus* or *B. multivorans* and incubated at 37ºC for 18 h. The production of anti-microbial agents was determined by the presence of a clearing zone surrounding *the Burkholderia* colony.

2.7 Effects of different factors on anti-candidal activity of *Burkholderia*

2.7.1 Effects of carbon, nitrogen, phosphate and temperature

To study the effect of carbon sources at different concentrations on anti-candidal activity of *Burkholderia*, the following carbon sources were evaluated in BSM at different concentrations (0, 10, 20, 40, 60 & 80 mM): glucose, glycerol, arabinose, fructose, galactose, sucrose, lactose, maltose, ribose, succinate and starch. Stock solutions of each carbon source were made and sterilised by autoclaving; this was then added aseptically to autoclaved, molten BSM agar made without any carbon source.

To determine the optimal conditions for anti-candidal activity of *Burkholderia*, seven different nitrogen sources were tested: beef extract (3g l^{-1}), yeast extract (0.5 g l^{-1}), peptone (2 g l^{-1}), urea (2 g l^{-1}), ammonium sulphate (4.89 g l^{-1}), ammonium chloride (2 g l^{-1}) and potassium nitrate (3.47 g l^{-1}). Three phosphate sources; dibasic potassium phosphate sodium phosphate and calcium phosphate at 1, 10, 40 and 80 mM were compared. HEPES ($C_8H_{18}N_2O_4S$) at 20 mM was used as buffer solution to maintain culture pH at 7.20. Seven

growth temperatures (10, 15, 20, 25, 30, 35 and 40°C) and four points of incubation (24, 72, 120 and 168 h) were evaluated.

To test all the growth parameter variables a standard antimicrobial production and overlay assay was performed (section 2.5.1), except that after growth of the Bcc isolates, the cells were harvested by centrifugation and resuspended in 3 ml of sterilised distilled water, prior to inoculation of each plate.

2.7.2 Effects of pH on growth rate and production of anti-candidal agents

To evaluate the effect of different pH of BSM-G and ISOB media on anti-candidal activity of *Burkholderia* and growth of *B. adusta* and *C. albicans*, the pH of the media was adjusted using 0.1 M HCl or 0.1 M NaOH as required to produce the following range 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. TSA, SA agar, TSB and SA broth (all at pH 7) were used as control media. For each test a 3 µl drop of *B. ambifaria* AMMD, BCF, JW13 and *B. vietnamiensis* Kc311 was spotted onto the centre of each BSM-G plates and incubated at 30ºC for 3 d. The effect of pH was evaluated using a *C. albicans* agar overlay assay as described in section 2.5.1. A control overlay with *C. albicans* was performed on all pH adjusted media to ensure the growth of this test organism was unaffected.

To examine the effect of pH on the secretion of *Burkholderia* antifungal active metabolites on the biosidiomycete, *B. adusta* BK1, its extension rate on BSM-G medium was determined at a range pH (pH 3.5 – 8.00). Each agar plate was inoculated at the centre with a 5 mm BK1 colonized agar plug (as described in section 2.6) and the plates incubated in the dark at 25ºC. The mycelial radius was measured (mm) after 1, 2, 3, 4, 5 and 6 d.

An automated microbial growth analyser, the Bioscreen C Microbiological Growth analyser (Lab systems, Finland) was used to monitor effects of different pH (range 3.5 - 8) on growth ability of *C. albicans* in a micro-well growth assay. ISOB medium (100 µl at different pH) and 100 µl of *C. albicans* inoculum (1 x 10^6 cfu ml^{-1} in ISOB) was added to each well of the 100-well Bioscreen plate. Plates were incubated in Bioscreen C bioanalyzer at 37°C, shaking 10 sec before the optical density was sampled every 5 min for up to 24 h, using wide range of fluorescence reader (optical density between 420-580 nm). The mean of 5 replicates was determined for each cultivation pH and plotted as a growth curve using Excel.

2.7.3 Time course of production and activity of anti-candidal agents by *B. ambifaria* AMMD

Changes in bacterial growth, culture pH and anti-candidal production over time were studied using BSM-G broth medium supplemented with glycerol (4 g l^{-1}). The pH was adjusted to pH 6 before autoclaving, and confirmed as the same after autoclaving. Each 250 ml flask contained 100 ml of BSM-G medium plus 50 µl of *B. ambifaria* AMMD as a standardised inoculum (Approximately 5 x 10^6 cfu ml^{-1}). The flasks were incubated statically and in shaker incubator at 30ºC. At 6, 14, 26, 38, 50, 74, 98, 122, 146 and 170 h mean bacterial growth (turbidity, using spectrophotometer at 630 nm) was determined from 3 replicates. Also, at each time point antimicrobial compounds were extracted and these activities were determined as follows. The bacterial culture was centrifuged for 10 min and the pH of the culture was determined by measuring the cleared supernatant. Bacterial secondary metabolites were then extracted from the supernatant using XAD16 resin as described below (see section 2.9.2). The *B. ambifaria* extracts were run on TLC

plates and overlaid with *C. albicans* to detect their *B. ambifaria* antifungal profile over time (section 2.10). BSM-G medium (without a bacterial inoculum) was used (6, 26 and 74 h time point) alongside the treatments as a control.

2.8 Transposon mutagenesis

2.8.1 Screening *B. ambifaria* AMMD Tn5 transposon mutant bank

A bank of 2500 *B. ambifaria* AMMD miniTn5-Km2 transposon mutants was created (Mahenthiralingam, *et al*., 2011) and screened for mutants which lack anti-candidal activity as follows. BSM-G plates containing the antibiotic kanamycin (50 µg ml^{-1}) were spotted with AMMD mutants over an eight point grid using a sterile toothpick; they were then incubated for 72 h at 30ºC. The bacterial growth was killed using chloroform and overlaid with soft Isosensitest agar seeded with *C. albicans* as described in section 2.5.1. The plates were incubated for 24 h at 37ºC and mutants which demonstrated alteration in their ability to produce anti-candidal agents noted. Individual mutants of interest were replated from the master bank of mutants that had been stored frozen as 96-well plate cultures. Each mutant was retested twice to confirm stability of their anti-candidal phenotype. Stable mutants were stored as TSB cultures supplemented with 8% DMSO at –80ºC until use.

2.8.2 Random amplified polymorphic DNA protocol (RAPD-PCR)

A RAPD-PCR fingerprinting protocol was performed as described by Mahenthiralingam, *et al*., (1996) to test whether each of the above transposon mutants was a derivative of *B. ambifaria* AMMD and not a contaminant that may have appeared during screening of the

mutant bank. The volume of each PCR was 25 µl and contained: 2 µl of genomic DNA (approximately 10 to 40 ng), 10.3 µl sterile nuclease free H_2O, 2.5 µl 10X Coralload buffer, 5 µl 5X Q-Solution, 1.5 $MgCl_2$, 0.5 µl 10 mM dNTPs, 4 µl primer 270 (5'- TGC GCG CGG G -3', 10 pmol μl^{-1}) or primer 272 (5'- AGC GGG CCA A -3', 10 pmol μl^{-1}) and Taq DNA polymerase (Qiagen Ltd., Carwley, UK). The PCR was carried out in 0.5 ml HI-YIELD micro tubes using a Flexigene thermal cycler (Techne Ltd., Newcastle, UK) as described in Table 2.5. 12 µl RAPD-PCR products of each sample were loaded to 2 % gel electrophoresis agarose gel, then run alongside 10 µl of 1 kb molecular ladder in 1X TBE buffer at 80 volts for 3 h. The gel was then stained with Save view (SYBR safe DNA gel stain, Invitrogen, Eugene, Oregon, USA), and visualised under UV light and images were taken using Gene Snap system.

2.8.3 Sequencing of Tn5 transposon mutant using PCR to detect transposon insertion site

PCR followed by DNA sequence analysis was used to determine the transposon insertion site in *B. ambifaria* AMMD genome as described by O'Sullivan, *et al*., 2007. DNA adjacent to the Tn5 insertion was amplified using a two-step of PCR reaction protocol (PCR1 and PCR2) and 4 different primers: PCR1: Primer 1: 5'- TTT TTA CAC TGA ATG TTC CG-3', Primer 2b: 5'- GGC CAC GCG TCG ACT AGT CAN NNN NNN NNN ACG CC-3', PCR2: Primer3: 5'- CGG ATT ACA GCC GGA TCC CCG-3' and Primer 4: 5'- GGC CAC GCG TCG ACT AGT AC-3'.

PCR 1 (including primers 1 & 2b) amplified the end of the transposon and adjacent chromosomal DNA to give sequences of variable lengths due to the random priming of primer 2b at genomic sites flanking the transposon. The volume of PCR1 reaction was 20

µl containing 1 µl genomic DNA (10 to 40 ng), 2.9 µl sterile nuclease free H_2O, 2 µl 10X buffer, 4 µl 5X Q- solution, 1.2 µl MgCl2, 0.4 µl 10 mM dNTPs mixture, 1 µl primer 1 (20 pmol μl^{-1}), 1 µl primer 2b (20 pmol μl^{-1}) and 0.2 µl Taq DNA polymerase. PCR was carried out in 0.2 ml non-stick micro-tubes using a DNA Engine Dyad Thermal cycler gradient block (MJ Research, Boston, MA) as described in Table 2.6.

Table 2.5 PCR cycles for RAPD-PCR.

Step	**Temp. Cº**	**Time (min.)**	**Nº cycles**
1. DNA denaturation	94	5	4
2. Primer annealing	36	5	
3. Primer extension	72	5	
4. DNA denaturation	94	1	30
5. Primer annealing	36	1	
6. Primer extension	72	2	
7. Final extension	72	6	1
8. Indefinite hold	4	0	0

Table 2.6 PCR cycles for PCRI of transposon mutant mapping.

Step	**Temp. Cº**	**Time**	**Nº cycles**
1. Initial DNA denaturation	95	00.05.00	1
2. DNA denaturation	94	00.00.30	8
3. Primer annealing	30	00.00.35	
4. Primer extension	72	00.00.45	
5. Initial DNA denaturation	95	00.05.00	1
6. DNA denaturation	94	00.00.30	30
7. Primer annealing	30	00.00.35	
8. Primer extension	72	00.00.45	
9. Indefinite hold	4	0	0

Products of PCR1 were further amplified using primers 3 and 4 in round PCR2 as follows. The volume of the PCR2 reaction was 50 µl containing: 2.5 µl PCR1 final product, 5 µl 10X PCR buffer, 10 µl 5X Q- solution, 3 µl $MgCl_2$, 0.4 µl 10 mM dNTPs mixture, 2.5 µl primer 3 (20 pmol μl^{-1}), 2.5 µl primer 4 (20 pmol μl^{-1}) and 0.5 µl Taq DNA polymerase (all PCR reagents from Qiagen Ltd. Carwley, UK). PCR processes were carried out in 0.2 ml non-stick micro-tubes using a DNA Engine Dyad Thermal cycler gradient block (MJ Research, Boston, MA) as described in Table 2.7. PCR2 products were purified using QIAquick PCR Purification Kit (Qiagen Ltd), then 10 µl of purified PCR2 products were loaded in 2% agarose gel supplemented with safe view dye (2.5 µl / 100 µl gel) and run alongside 10 µl 1-kb molecular ladder. Gel electrophoresis was run at 80 volts in 1X TBE buffer containing save view dye (2.5 µl / 100 ml TBE) then the gel was visualised under UV light and images were taken using Gene Snap system. PCR2 products (<13 ng μl^{-1}) and primer 3 (1.6 pmol μl^{-1}) were sequenced, and the identities of putative genes that were disrupted by transposon insertion were identified using the *B. ambifaria* AMMD genome (http://www.Burkholderia.com) and BLASTN tool.

Table 2.7 PCR cycles for PCRII of transposon mutant mapping

Step	**Temp. C°**	**Time**	**N° cycles**
1. Initial DNA denaturation	95	00.02.00	1
2. DNA denaturation	94	00.00.30	30
3. Primer annealing	42	00.00.35	
4. Primer extension	72	00.00.45	
5. Final extension	72	00.05.00	1
6. Indefinite hold	4	0	0

2.9 Extraction of antimicrobial agents, storage and maintenance

Antimicrobial agents of Bcc bacteria were extracted in two different ways: Amberlite XAD16 resin extraction and direct extraction.

2.9.1 Maceration (Direct) extraction

Burkholderia isolates were inoculated as strips of culture across the agar plates using a sterile cotton swab (1 to 3 strips per plate) then incubated at 30C for 72 h and the bacterial growth was cut and removed from each plate using a clean scalpel blade. The remaining agar was frozen at -80ºC and then placed in a freeze dryer for 72 h until the agar had completely dried into thin films. The dried agar was ground to fine particles using a coffee grinder. The agar particles were then extracted in 80% methanol (HPLC grade) at ratio 8 g agar per 45 ml 80% MeOH; extractions were performed in 50 ml conical tubes and were left mixing on a rocking platform at room temperature (approximately 20 ± 2ºC) for 3 h. After extraction the tubes were centrifuged for 10 min, the supernatant was removed and the extract was concentrated using a rotary evaporator (Buchi, Flawil, Switzerland) at 30ºC until the volume of the extract reduced approximately 10 times. Concentrated extracts were stored into micro-tubes at -20ºC until use.

2.9.2 Resin extraction

Amberlite, a neutral cross-linked polystyrene resin that adsorbs erythromycin from solution (Ribeiro & Ribeiro, 2003) was also used as a means to purify secreted antibiotics from agar and broth cultures of *Burkholderia* as follows: strips of *Burkholderia* growth were inoculated onto each plate (3 to 5 strips across the agar) and left to grow for the desired length of time at a specific growth temperature (see results 4.3.1 for conditions

tested). A total of 12 plates were used for standard extractions. To extract the antifungal components from the agar, the bacterial growth was removed by swapping with a spatula and the remaining treated with chloroform vapour. The agar was then cut into 0.5 cm blocks using a clean scalpel blade and placed into a large beaker. For every 100 g agar, 500 ml polished water was added and the mixture was stirred for 1 h at room temperature. The mixture was then filtered to remove agar blocks and Amberlite XAD16 resin was added to the aqueous extract (5 g /100ml) and stirred for 2 h at room temperature to allow antibiotics to bind to the resin. The resin was then allowed to settle out and the supernatant discarded; the resin was rinsed with 1 l of polished water. A resin column was prepared by inserting a loose glass wool plug into the bottom of a glass column (60 mm long x 25 mm diameter) and the resin slurry was poured into the column; compressed air was used to remove water residues from the resin supernatant mixture. To elute the *Burkholderia* metabolites from resin, 100% methanol was used (3ml methanol/1g of resin). The extracts were concentrated by evaporation of the methanol using a rotary evaporator machine at 30ºC until dry; after weighing of the dried extracts, they were dissolved in methanol (normally at concentration of 10 mg ml^{-1}) and stored in micro-tubes at -20ºC until use.

To extract *Burkholderia* antibiotics from liquid cultures, the Amberlite resin procedure was modified as follows. Liquid cultures (normally 100 ml) were inoculated and incubated under different growth conditions as required. After growth, the bacteria were removed by centrifugation (Beckman Coutere centrifuge, Sorvall Avanti J-E). The Amberlite resin was then added to the cleared supernatant and processed to extract the antimicrobials exactly as described above for the agar plate cultures.

2.10 Thin layer chromatography and bioautography assay

2.10.1 Thin layer chromatography

Thin layer chromatography (TLC) was carried out to fractionate compound(s) in *Burkholderia* secondary metabolite extracts; a bioautography assay was developed to detect any bioactive compounds in this mixture. Two different types of TLC plate were used: 20 x 20 cm pre-coated 25 µm Silica Gel 60 plates (Sigma, catalogue no. 5715; Merck) and 20 x 20 cm pre-coated 25 µm Silica Gel 60 plates with fluorescent indicator UV 254 (Sigma, catalogue no. 5715; Merck); both plate types were activated by heating at 100°C for 60 min. 5 µl of *Burkholderia* extracts (10 mg ml^{-1}) were spotted onto the TLC plates 2 cm from the base across a horizontal baseline and at least 2 cm apart; a maximum of 8 extracts were evaluated on any 20 cm plate and replicate plates were frequently set up to enable bioactivity testing with multiple test species. Different solvents (single and mixture solvents with different polarities) were tested to generate the best possible fractionation profile of Bcc secondary metabolites. The plates were developed vertically in a glass chromatography tank containing 200 ml of solvent until the solvent front reached the top of the TLC plate, 2 cm from top edge.

Several visualisation techniques were used to monitor developed TLC plates. The developed TLC plates were allowed to dry and freeing of solvent. The first visualisation technique was iodination. Iodination was carried out by placing a dried developed TLC plate in a chamber containing iodine crystals for few minutes, which led to oxidization of the substances making their presence visible. The second visualisation technique used a different chemical solution (0.1% Anisaldehyde–sulfuric acid, 0.2% Ninhydrin, 0.5% Vanillin-Phosphoric acid, or 25% sulphuric acid). In this method the dried developed TLC plates were sprayed with a freshly prepared solution for 15 min then placed in a 100ºC

oven for 30 min to 1 h, in contact with the substances a visualisation solution exhibited a coloured spots of extract constituents. The third visualisation technique used UV radiation at two different wave lengths (254 and 366 nm); normal and fluorescent TLC plates were visualized under UV light by placing the silica gel side in front of the UV source, which provided a rapid and straightforward screen to identify the majority of compounds. Pure pyrrolnitrin antibiotic (3-Chloro-4-[3-chloro-2-nitrophenyl]-pyrrole; Sigma, P8861) was used as a positive reference compound against new active compounds. Extracts of growth media (BSM-G media and SA extracts) incubated without *Burkholderia* isolates were used as a negative controls. The distances travelled by visualized or active compounds relative to the solvent front were determined as a retention time (R_f). Developed plates were photographed using a digital camera (Sony DSC-W5).

2.10.2 Bioautography

To reveal the presence of bioactive *Burkholderia* secondary metabolites, the following test organisms were overlaid onto the TLC plates: Gram-negative bacteria (*E. coli* NCTC 12241, *B. multivorans* ATCC17210, *B. cenocepacia* LMG16656, *P. aeruginosa* NCTC12903, *P. putida* LMG 2257, *P. fluorescens, S. maltophilia, A. baumannii* and *E. faecalis*), Gram-positive bacteria (*Staphylococcus aureus* NCTC12981, other multidrug resistance *S. aureus* Eagles, *S. aureus* EMRSA15, and *S. aureus* MRSA Mu50), an ascomycete fungus (*C. albicans*) and basidiomycetes fungi (*H. fasciculare*, *P. impudicus*, *B. adusta*, *S. gausapatum*, *T. versicolor*, *R. bicolor* and *P. velutina*). *Burkholderia* extracts were applied to 20 x 20 cm TLC plates (0.25 mm silica gel–plate) as described above and fractionated in a glass chromatography tank containing 200 ml of ethyl acetate-methanol-water (EtOAc-MeOH- H2O; 20:1:0.5). One set of TLC plates was developed chemically or

photographed under UV for use as the reference chromatogram; the remaining replicate plates were set aside for the bioassay work as follows.

The TLC plate was placed into a sterile 24 x 24 cm square Bioassay dishes (Sterilin, UK). The test organism (*Candida* and all bacteria) was inoculated into molten SISO agar (cooled to 45^{o}C) with 2,3,5-triphenyltetrazolium chloride (TTC) added to 0.02% to assist with visualisation of microbial growth. After mixing, the molten agar was poured (75 ml/ plate) carefully over the TLC plate starting in the centre and then moving evenly across the entire plate. After solidification, the TLC-bioautographic plate was incubated for 3 h at room temperature to allow metabolites in the TLC plate to diffuse into the agar; the bioassay plate was then incubated for 24 h at 37°C to allow microbial growth. Clear zones on the red background of microbial growth revealed the presence of antimicrobial compounds. Growth inhibition areas were compared with the R_f of the related spots on the reference TLC plate and all plates were photographed under white light using a digital camera.

In the case of the basidiomycete overlay assay, the developed TLC plate was overlaid with soft SA agar (0.75% agar) seeded with the fragmented mycelia of each fungus (see section 2.5.2.2) and the plates were then incubated at 20°C for 4 d. To help visualize fungal growth the plates were covered with 2 ml of 2% TTC. Anti-basidiomycete activity was detected as a clear zone on the red background of basidiomycete mycelia growth.

2.11 Purification of active metabolites of *Burkholderia*

To increase the purity of *Burkholderia* extracts available for further biological and chemical analysis, a wide range of separation techniques including liquid fractionation,

silica gel preparative thin layer chromatography and silica gel column chromatography were used. Active fractions obtained from these preparative methods were tested on the bioassay as described above with *C. albicans* used as the main test organism. The following extraction methods were used.

2.11.1 Liquid-liquid fractionation

A liquid-liquid solvent partition method was used to further extract the *Burkholderia* Amberlite resin mixtures. A dry crude methanol extract (approximately 2.5 g) of *B. ambifaria* AMMD was dissolved in 50 ml of distilled water and successively partitioned three times with a range of solvents (50 ml of solvent for each extraction). A fractionation scheme was employed as shown in Figure 2.3. Each fractionation involved hand-mixing of aqueous crude extract in the respective solvent followed by soaking at 25ºC for 15 min.

The aqueous crude extract and the various solvent fractions (diethyl ether fraction [DEF], chloroform fraction [CHF], dichloromethane fraction [DHF], acetone fraction [ACF], ethyl acetate fraction [EAF] and water fraction [WF]) were filtered through Whitman no.1 filter paper. The fractions were dried using a rotary evaporator machine at 30ºC. The dry weight yield of each extract and its fractions was measured before storage at -20ºC prior to further analysis.

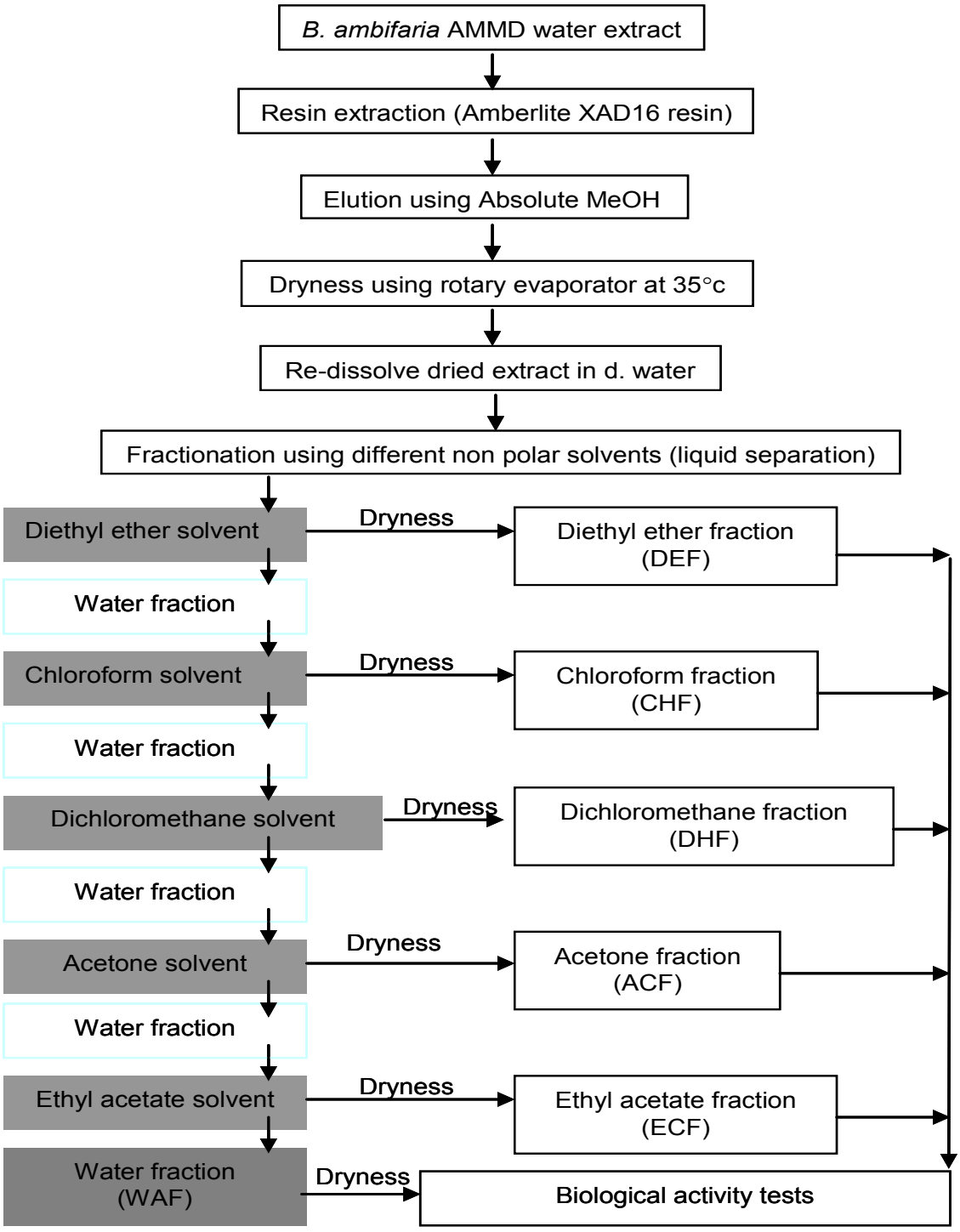


Figure 2.3 Scheme for fractionation of *B. ambifaria* AMMD antimicrobial extracts.

2.11.2 Preparative silica gel TLC plates

Dried extracts of *B. ambifaria* AMMD, BCF, KW and Mex-5 that had antimicrobial activity were re-dissolved separately in methanol and subjected to preparative TLC on 500, and 1500 µm Silica Gel 60 plates (Sigma, catalogue no. 5715; Merck). After inoculation of the extracts the mixture were fractionated with EtOAc-MeOH-H_2O (20:1:0.5). The location (R_f) of active constituents of each extract was detected directly on a replica TLC plate which was subsequently subjected to bioassay with the *C. albicans*. The preparative TLC plates were divided into 7 to 16 horizontal zones depending on the number of detectable active compounds after fractionation. Each zone was scraped off the plate and the constituents re-extracted from the silica gel using absolute MeOH. The resulting fractions were evaporated to dryness in rotary evaporator at 30ºC and retested for activity against *C. albicans* using agar diffusion assay on TLC plates to confirm their bioactivity after fractionation. The semi-purified extracts were kept at -20ºC for further analysis.

2.11.3 Column chromatography

Dried *B. ambifaria* AMMD aqueous extract (3 g) was re-dissolved in ethyl acetate and applied to a column chromatograph (5 x 50 cm) that had been packed with silica gel (70-230 mesh, 40 Å) and previously equilibrated with ethyl acetate methanol: water (EtOAc-MeOH-H_2O) 20:1:0.5v/v. The column was eluted 300 ml of EtOAc-MeOH-H_2O to produce 59 fractions (5 ml/fractions), then the column was eluted with 50 ml of absolute methanol to produce 4 fractions of AMMD extract. The different fractions eluting from the column were concentrated by evaporation of the solvent using a rotary evaporator machine at 30ºC, and re-tested for activity using pre-coated 25 µm Silica Gel 60 plates and overlaid with *C. albicans* to detect active fractions. Semi pure extracts were merged and kept at –20ºC for further analysis.

2.12 Identification bioactive compounds of *Burkholderia*

2.12.1 Using reversed phase liquid-chromatography-mass spectroscopy (RP-LC-MS)

High performance Liquid Chromatography (LC) coupled with Mass spectrometry (LC-MS) provides highly detailed information about product ions and is one of the most rapid sensitive methods for the detection and putative identification of bioactive organic compounds. Active Bcc fractions isolated as described above were chemically analysed and identified using Reversed Phase-Liquid chromatography-mass spectroscopy (RP-LC-MS) that was collaboratively performed by Dr. Lijiang Song (University of Warwick, Coventry, UK). LC performed on trial and error challenge was needed to optimize the best conditions of extraction, fractionation and visualization.

A Dionex 3000RS with a Sigma Sulpeco Accentis Express column (C18, size: 150 x 2.1mm, particle size: 2.7um) was used with the following mobile phases: **A**: H_2O with 0.1% formic acid; **B**: MeOH with 0.1% formic acid (a gradient from 0 to 20 min was run from 20% solvent **A to** 100% solvent **B)**. The UV spectra of bioactive *Burkholderia* compounds were compared with retention times of UV spectral data library of antibiotics and natural products to identify matches to known compounds. To provide further information on the structure of the *Burkholderia* antimicrobials, Nuclear Magnetic Resonance (NMR) spectroscopy was performed collaboratively on the RP-LC-MS fractions by Dr. Song using a Bruker 700 MHz NMR spectrometer.

2.12.2 Detection of the presence of pyrrolnitrin in *Burkholderia* metabolites: a comparative study

Many of the previous studies on *Burkholderia* sp confirmed that the key role of antifungal activity was due to different compounds e.g. pyrrolnitrin. Therefore, in this study pure pyrrolnitrin (Sigma) was included as a positive control compounds for *Burkholderia* anti-candidal metabolites. Burkhead, *et al*. (1994) and Schmidt, *et al*. (2009) recommended different conditions to isolation and detection of pyrrolnitrin.

B. ambifaria AMMD fraction no. 7 (R*f* 0.98) of the silica gel preparative TLC fractionation (see section 4.3.4.1), *B. ambifaria* AMMD first (G1), third (G3) and fourth (G4) fractions of silica gel column chromatography (see section 4.3.4.3), AMMD acetone extract which was extracted by liquid extraction using acetone, last fractions of silica gel preparative TLC fractionation of BCF, KW and Mex-5 (see section 4.3.4.1; 5 µl of 10 mg ml^{-1}) and 5µg pure pyrrolnitrin (sigma) were used and run in duplicate and compared side by side on the same plate. The first run of these fractions and standard compound were carried out on normal 0.25 silica gel TLC plates and chloroform, ethyl acetate and formic acid 5:4:1v/v as a solvent system (Burkhead, *et al*., 1994). The second run was carried out on florescence 0.25 silica gel TLC plates and chloroform and acetone 9:1v/v (Schmidt, *et al*., 2009). One set of both was visualized under UV light at 366 nm and the other set was overlaid with *C. albicans*.

2.13 Statistical analysis

All data were analysed using MINITAB 16 software. For examining the effect of media type on extension rate (mm/h) of cord forming, wood decay basidiomycetes, analysis of covariance (General linear model) was used followed by comparisons Tukey tests at the

95% confidence level ($P \leq 0.05$). To evaluate the effect of media type and media composition on production of anti-candidal agents, one way and two way analysis of variance (ANOVA) were used followed by comparisons (Tukey test) at the 95% confidence level ($P \leq 0.05$). Unless otherwise stated, data are presented with means and standard errors of the mean.

CHAPTER THREE

A SYSTEMATIC SCREEN OF ANTIFUNGAL ACTIVITY OF *Burkholderia cepacia* complex AND OTHER *Burkholderia* SPECIES

3.1 Introduction

Burkholderia species inhabit a large array of ecological niches and members of the genus are known to be plant, human and animal pathogens. *Burkholderia* exist in soil, water and plant rhizospheres and can be endophytically associated with fungi (Coenye & Vandamme, 2003).

Basidiomycetes are major agents of decomposition, which may attack standing and felled timber, stumps and other man-made wooden structures. *C. albicans* is the major opportunistic fungal pathogen causing potentially fatal disease in immunosuppressed human hosts (Pfaller & Diekema, 2007). In this study, ten representatives of basidiomycete and *C. albicans* were used as target fungi to screen for antifungal compounds.

The interaction between antibiotic producing bacteria and fungi especially phytopathogens, has been studied by many researchers for a long time, especially in terms of the biopesticidal uses for agriculture, and they attach a particular significance to the metabolites produced during interactions, which have potential as antimicrobial agents (Kadir*, et al.*, 2008, Rousk*, et al.*, 2008, Sultan*, et al.*, 2008). To date only limited attention has been given to interactions between wood decay basidiomycetes and bacteria, especially *Burkholderia*.

There are many Bcc strains known as antimicrobial producers that have been characterized as bio-pesticides (Parke & Gurian-Sherman, 2001). Nearly all of Bcc metabolites exhibited antimicrobial activities were identified from *"Burkholderia cepacia"* isolates which have

not been characterized at the species level in terms of the Bcc, and no studies have looked at large collections of taxonomically-defined Bcc isolates.

The overall aim of this work was to perform a systematic screen of *Burkholderia* antifungal activity using a large collection of accurately identified *Burkholderia* species. This was performed using different growth media and using both an overlay assay and direct antagonism assay to test antimicrobial secretion and inhibition. An initial challenge of the work was that the bacterial-fungal interaction media used should support both fungal and bacterial growth and aid the production of antimicrobial secondary metabolites. The type of media influences the interaction between bacteria and fungi as these organisms differ in responses to abiotic factors, such as nutritional requirements, temperature and hydrogen ion concentration (Hwang*, et al.*, 2002, Murray & Woodward, 2003, Li*, et al.*, 2007, Kadir*, et al.*, 2008). Various media formulations were therefore tested prior to the optimal media being selected to examine the diversity of anti-fungal activity across a collection of 421 *Burkholderia* isolates.

An additional challenge of the work was using wood-decay basidiomycete fungi which, unlike the ascomycete *Candida* species, grow as mycelia, and thus are not as straightforward to screen in antimicrobial assays. Their interaction with *Burkholderia* species has not been extensively examined. A conventional contact antagonism assay applied to many filamentous fungi was used as an initial screen, however, a novel overlay assay where homogenised mycelia were used to form an even growth of the basidiomycete was also developed.

A general hypothesis for this chapter was "**all *B. cepacia* bacteria are capable of producing antifungal agents"**. The specific aims of this chapter were:

- To evaluate a range of growth media for their ability to support fungal and bacterial growth, and allow the production of antimicrobial metabolites.
- To systematically screen a collection of *Burkholderia cepacia* complex species for the production of antifungal metabolites.
- To examine the secretion of antifungal metabolites in *Burkholderia* species outside the complex and compare this to a range of non-*Burkholderia* species.
- To develop an overlay-inhibition assay suitable for screening antifungal activity against filamentous basidiomycetes.

3.2 Materials and Methods

3.2.1 Growth media and microorganism storage and maintenance

The media used in the current experiment were as described in section 2.1. A total of 421 bacterial isolates (section 2.1), including cystic fibrosis, clinical and environmental isolates (plant pathogenic and soil isolates), were screened against *Candida albicans* Sc 5314 and *B. adusta* BK1. *C. albicans* (1 X 10^{-6} cfu ml^{-1} overlay agar) was used as a reference strain to rapidly test the activity of antifungal agents produced by the *Burkholderia* isolates. Ten representative species of wood decay basidiomycete fungi (Table 2.2) were used to detect the activity against this class of fungi. A basidiomycete inoculum was aseptically transferred onto a slant culture tube containing 2% MA medium as described in section 2.1.

3.2.2 Selection of suitable media for growth and antifungal metabolite production

Four Bcc isolates (*B. ambifaria* AMMD, *B. ambifaria* BCF, *B. ambifaria* Kc311 and *B. vietnamiensis* JW13) that were known to produce anti-candidal agents were tested for the production of anti-candidal agents on six different media, (SA, PDA, MA, BSM-G, CMA and MHA), using a *C. albicans* overlay assay as described in Section 2.4. The production of anti-candidal agents was determined by measuring the zone of inhibition in mm. To examine the affect of different media on basidiomycete fungi, the growth rates of ten wood decay fungi were examined on seven different media (SA, PDA, MA, BSM-G, TSA, ISOA and MHA); growth was determined at 3, 5, 7, 9 and 11 d by measuring the hyphal extension rates in millimetres (mm; see section 2.6).

3.2.3 Interactions between *Burkholderia* and fungi

After the selection of appropriate media (3.2.1 & 3.2.2), a total of 421 bacterial isolates (section 2.1) were screened against *C. albicans* and *B. adusta* BK1 using SA and BSM-G media. The interaction between *Burkholderia* and *C. albicans* was carried out using an overlay agar assay (section 2.5.1) and the results were determined by measuring the zone of inhibition in mm. The interaction between *Burkholderia* and *B. adusta* were carried out using a contact antagonism assay (section 2.5.2.1) and the results scored by comparing to the BK1 control plates, noting whether there was: no inhibition (-), weak inhibition [(+); 0.1 cm to 0.5 cm of no BK1 growth], medium inhibition [(++); 0.5 cm to 1 cm of no BK1 growth] or strong inhibition [(+++); 1 cm to 2 cm of no BK1 growth] of fungal growth by *Burkholderia*. Eight wood decay basidiomycetes were also tested against four *B. ambifaria:* AMMD, BCF, KW and MEX-5 using a basidiomycete agar overlay assay as described in section 2.5.2.2.

Peptone Lactose Agar medium (PLA), which was shown to enhance the production of antifungal agents of putative *B. multivorans* isolates in another study (Dikin*, et al.*, 2007) was also used to test 27 isolates of *B. multivorans* and 18 isolates of *B. stabilis*. These two Bcc species had totally failed to produce antifungal agents when grown on SA or BSM-G media, prompting the testing of this alternative medium.

3.2.4 Statistical analysis

All data were analysed using MINITAB 16 software. To examine the effect of media type on growth rate (mm/h) of wood decay basidiomycetes, the results were tested statistically using analysis of covariance (General Linear Model) followed by comparisons (Tukey Test) at the 95% confidence level ($P \leq 0.05$). To evaluate the effect of media type on the

production of anti-candidal agents, one-way analysis of variance followed by comparisons (Tukey test) at the 95% confidence level ($P \leq 0.05$) were used. Chi Square (χ^2) at the 95% confidence level ($P \leq 0.05$) was used to compare the effect of BSM-G and SA media on the production of anti-candidal agents and to test the effect of SA medium on the production of antifungal agents of the whole collection and between groups. All data are presented with means and standard errors of the mean.

3.3 Results

3.3.1 Selection of a suitable medium for growth and production of anti-microbial agents

3.3.1.1 Selection of growth media for wood decay basidiomycetes

In this study, different media (Table 2.1) were used for growing selected wood decay fungi (Table 2.3) and *Burkholderia* isolates (Table 2.2). Seven different media were used to test the growth ability of ten different wood decay fungi at 25ºC. Potato Dextrose Agar (PDA) was the optimal growth medium supporting the growth of all 8 basidiomycete fungi with growth rates ranging from 0.0 to 12.48 mm d^{-1} (Table 3.1, Figure 3.1). Sabouraud Agar (SA) and Malt Agar (MA, 2%) also supported the growth of basidiomycetes well, with only *P. impudicus* and *R. bicolor* failing to grow on each respective medium (Table 3.1). The remaining media tested showed weak growth for the wood decay basidiomycetes with many species failing to grow (Table 3.1, Figure 3.1). In rank order, the most rapid growing basidiomycetes were *B. adusta*, *T. versicolor* and *S. gausapatum* with growth rates of 14.8, 7.97 and 7.18 mm d^{-1} respectively, seen on Sabouraud agar (Figure 3.2).

The effects of different media on fungal growth were highly variable (Table 3.1, Figure 3.1). *B. adusta* showed the best growth rate on MA, SA and PDA media (Table 3.1, Figure 3.2) with the mycelia reaching the edge of the Petri dishes within 7 days. In contrast, *B. adusta* exhibited the weakest growth on BSM-G (1.37 mm d^{-1}) and only grew after 3 days of inoculation on ISA and MHA media. The effect of different media on fungal growth

Table 3.1 Effects of media type on extension rate of wood decay basidiomycetes.

		Extension rate (mm d^{-1} (SEM)) of wood decay basidiomycetes [a]									
		Sa1	**HfGTW**	**Rbm6A**	**HfDD2**	**Rb1**	**BK1**	**Pv1**	**PiJHT4**	**D2**	**SP313**
Media [b]	**MHA**	**1.28 (0.07)**	**0.0 (0.0)**	**0.0 (0.0)**	**0.0 (0.0)**	**0.0 (0.0)**	**8.47 (0.12)**	**0.0 (0.0)**	**0.0 (0.0)**	**3.72 (0.16)**	**0.0 (0.0)**
	ISO	**2.57 (0.06)**	**0.0 (0.0)**	**0.0 (0.0)**	**0.0 (0.0)**	**0.0 (0.0)**	**8.18 (0.15)**	**0.0 (0.0)**	**0.0 (0.0)**	**3.72 (0.14)**	**0.0 (0.0)**
	MA	**6.68 (0.14)**	**4.8 (0.17)**	**3.05 (0.0)**	**3.03 (0.18)**	**5.22 (0.09)**	**15.0 (0.14)**	**6.05 (0.13)**	**0.0 (0.0)**	**5.03 (0.14)**	**4.77 (0.07)**
	SA	**7.18 (0.07)**	**2.5 (0.08)**	**2.15 (0.09)**	**2.15(0.05)**	**3.15 (0.13)**	**14.8 (0.17)**	**1.68 (0.1)**	**3.0 (0.08)**	**7.97 (0.64)**	**5.7 (0.13)**
	TSA	**1.32 (0.07)**	**0.0 (0.0)**	**0.0 (0.0)**	**0.0 (0.0)**	**0.0 (0.0)**	**6.37 (0.32)**	**0.0 (0.0)**	**0.0 (0.0)**	**2.28 (0.1)**	**0.0 (0.0)**
	BSM_G	**0.0 (0.0)**	**0.0 (0.0)**	**0.0 (0.0)**	**0.0 (0.0)**	**0.0 (0.0)**	**1.37 (0.14)**	**0.0 (0.0)**	**0.0 (0.0)**	**1.88 (0.4)**	**0.0 (0.0)**
	PDA	**7.33 (0.2)**	**3.78 (0.02)**	**3.85 (0.04)**	**3.85 (0.5)**	**0.0 (0.0)**	**12.42 (.08)**	**9.77 (0.02)**	**4.25 (0.10)**	**11.9 (0.22)**	**3.95 (0.13)**

[a] *Stereum gausapatum* Sa1, *Hypholoma fasciculare* (HfGTW), *Resinicium bicolor* (Rbm6A), *H. fasciculare* (HfDD2), *R. bicolor* (Rb1), *Bjerkandera adusta* (BK1), *Phanerochaete velutina* (Pv1), *Phallus impudicus* (PiJHT4), *Trametes versicolor* (D2) & *Heterobasidion annosum* (SP313).

[b] Muller Hinton agar (MHA), Tryptone Soya agar (TSA), Isosensitest 0.75agar (ISOA), Basal salts media with glycerol (BSM-G), Potato dextrose agar (PDA), 2% Malt agar (MA) & Sabouraud agar (SA).

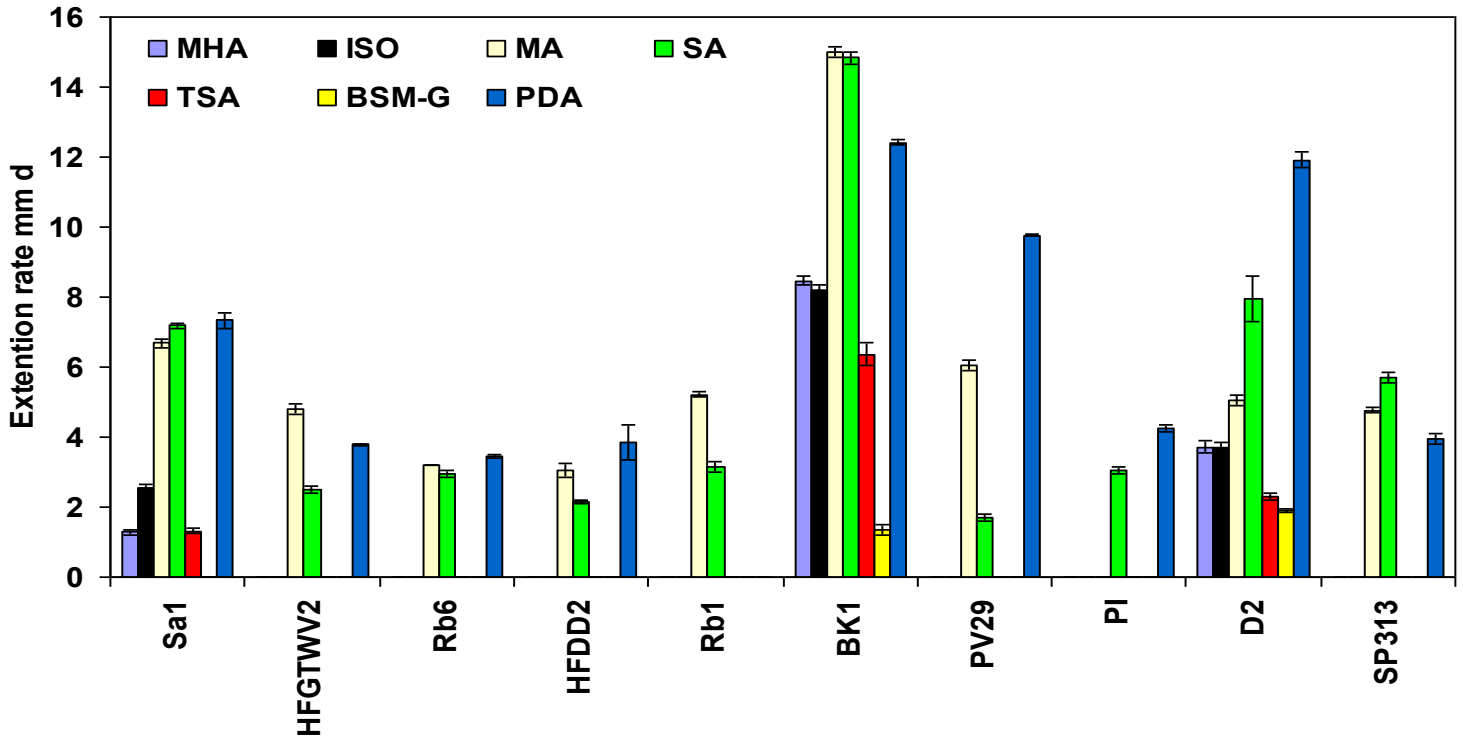


Figure 3.1 Effects of media type on extension rate mm d^{-1} (SEM) of wood decay basidiomycetes.

The growth rate was determined for the following species: *Stereum gausapatum* Sa1, *Hypholoma fasciculare* (HfGTW), *Resinicium bicolor* (Rbm), *H. fasciculare* (HfDD2), *R. bicolor* (Rb1), *Bjerkandera adusta* (BK1), *Phanerochaete velutina* (Pv1), *Phallus impudicus* (PiJHT4), *Trametes versicolor* (D2) & *Heterobasidion annosum* (SP313). The following media were examined: Muller Hinton Agar (MHA), Tryptone Soya Agar (TSA), Isosensitest 0.75 Agar (ISOA), Basal Salts Media with glycerol (BSM-G), Potato Dextrose Agar (PDA), 2% Malt Agar (MA) and Sabouraud Agar (SA).

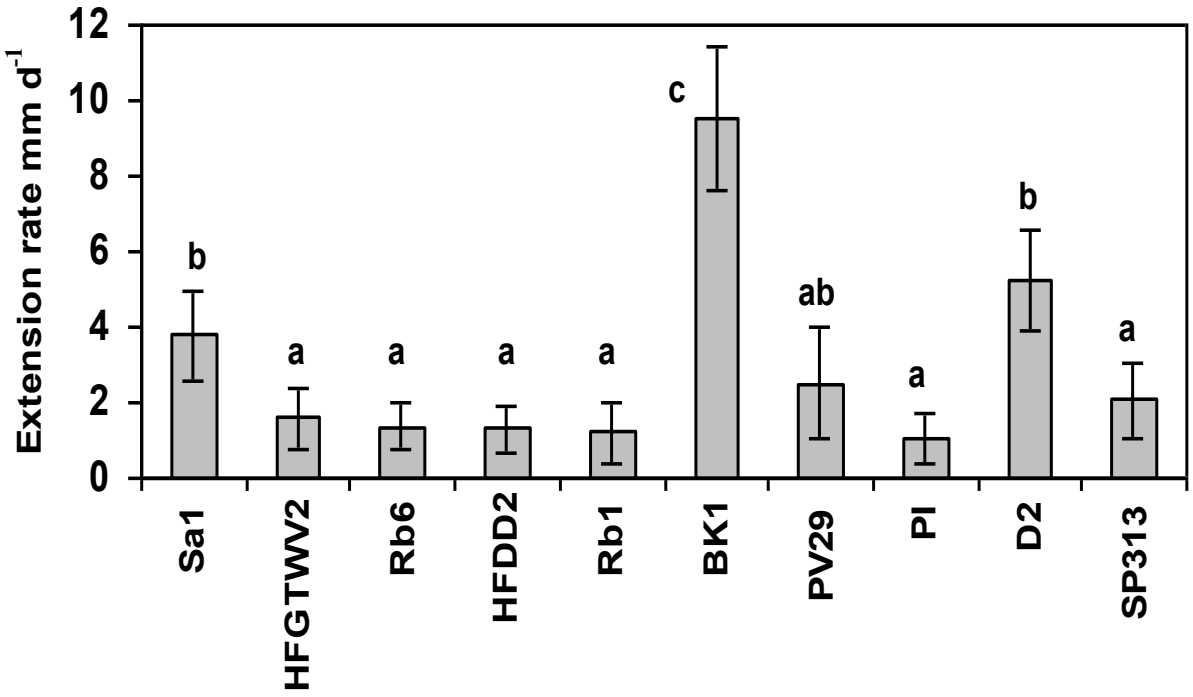


Figure 3.2 Mean extension rate mm d^{-1} of wood decay basidiomycete across all media.

Stereum gausapatum (Sa1), *Hypholoma fasciculare* (HfGTW), *Resinicium bicolor* (Rbm), *H. fasciculare* (HfDD2), *R. bicolor* (Rb1), *Bjerkandera adusta* (BK1), *Phanerochaete velutina* (Pv1), *Phallus impudicus* (PiJHT4), *Trametes versicolor* (D2) & *Heterobasidion annosum* (SP313). The following media were examined: MHA, TSA, BSM-G, PDA, MA and SA. Different letters above columns indicate significant differences (ANOVA; $P \leq 0.05$). Data are presented as the means of three replicates with standard errors of the means.

was highly significant ($P \leq 0.001$, F = 194.26), and all tested isolates showed (Figure 3.2, 3.3) a significant difference ($P \leq 0.001$) in production of anti-candidal agents.

T. versicolor showed the faster extension on PDA and SA media (11.9 and 7.97 mm d^{-1} respectively) and mycelia reached the edge of Petri dishes of PDA medium within 7 days. In contrast, *T. versicolor* showed the weakest growth on BSM-G, TSA, ISA and MHA media respectively and grew after 3 days of inoculation. *S. gausapatum* showed the faster extension on PDA, SA and MA (7.33, 7.18 and 6.68 mm d^{-1} respectively; Table 3.1). The mycelia reached the edge of PDA Petri dishes within 9 days, and grew after seven days on TSA and MHA. However, the fungus failed to grow on BSM-G medium.

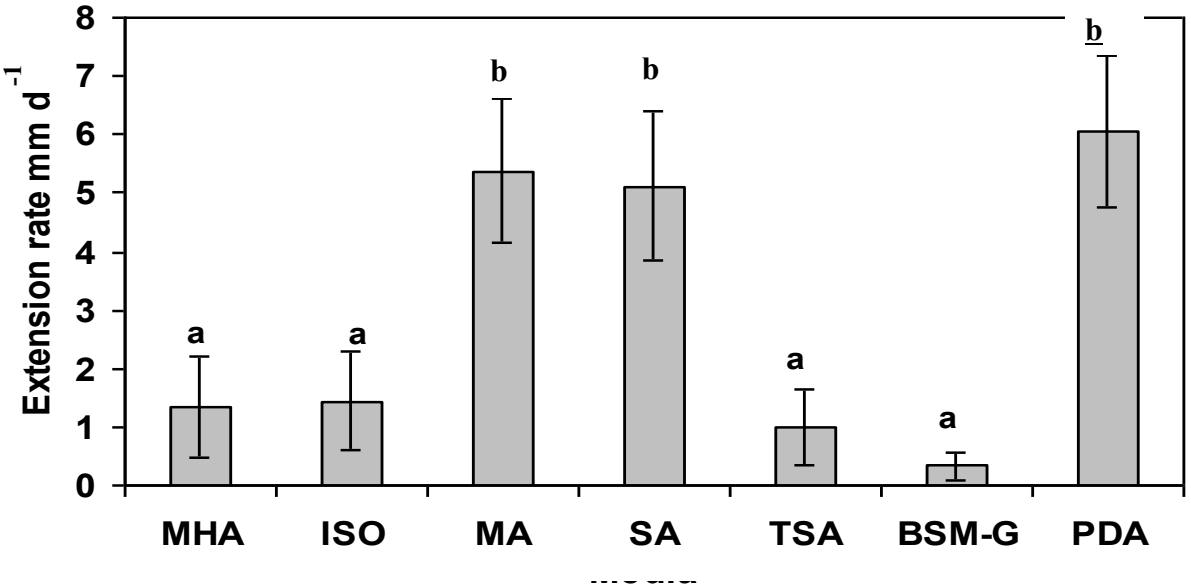


Figure 3.3 Effects of media type on extension rate mm d^{-1} of wood decay basidiomycetes.

Muller Hinton Agar (MHA), Tryptone Soya Agar (TSA), Isosensitest 0.75 Agar (ISOA), Basal Salts Media with glycerol (BSM-G), Potato Dextrose Agar (PDA), 2% Malt Agar (MA) and Sabouraud Agar (SA). Different letters above columns indicate significant differences (ANOVA; $P \leq 0.05$). Data are presented as the means of three replicates with standard errors of the means.

The effect of different media on fungal growth (Figure 3.2) was highly significant ($P \leq 0.005$, F = 98.72). *H. fasciculare* HFDD2 and HFGTW showed similar behavioural patterns on the same growth media, with for example the faster extension on PDA medium (3.85 and 3.78 mm d^{-1} respectively). The mycelia of both fungi covered about half of the Petri dishes within 11 days. Both *H. fasciculare* isolates showed weak growth on MA and SA media, and failed to grow on MHA, TSA, ISOA and BSM-G media.

The effect of different media on fungal growth was highly significant. Different strains of *R. bicolor* (Rb1 and Rbm6A) behaved differently. Rbm6A showed faster extension on PDA, MA and SA media (3.85, 3.05 and 2.15 mm d^{-1} respectively) but failed to grow on BSM-G and other media ($P \leq 0.001$, F = 3.01), whereas Rb1 extended faster on MA and SA media (5.22 and 3.15 mm d^{-1} respectively) and failed to grow on MHA, ISO, TSA, BSM-G and PDA media ($P \leq 0.005$, F = 154.95). *P. velutina* and *H. annosum* showed

similar activity, as they failed to grow on MHA, TSA, ISOA and BSM-G media. *P. velutina* grew well on PDA medium and the mycelium covered half of the Petri dish within 9 days (9.77mm d^{-1}; $P \leq 0.001$, F = 104.41) and also grew on MA. *H. annosum* grew on three different media: SA, MA and PDA ($P \leq 0.001$, F = 12.50). *P. impudicus* failed to grow on most of the tested media (MHA, TSA, ISO and BSM-G) and extended faster on PDA (4.25 mm d^{-1}; $P \leq 0.001$, F = 45.97).

3.3.1.2 Selection of growth media for anti-candidal production by *Burkholderia*

Six different media were chosen to test the production of anti-candidal agents from four Bcc isolates: *B. ambifaria* AMMD, *B. ambifaria* BCF, *B. ambifaria* Kc311 and *B. vietnamiensis* JW13. These strains are known producers of antibacterial agents (Mahenthiralingam *et al*. unpublished data), and production of anti-candidal agents was tested using an agar overlay assay.

The effects of *Burkholderia* metabolites on *C. albicans* were different from isolate to isolate and in additional different classes of interaction were observed. In the first class of interaction, no inhibition of *C. albicans* growth was observed (Figure 3.4 A). In the second class of interaction, clear growth inhibition occurred, with *Burkholderia* metabolites stopping the growth of *C. albicans*, resulting in a visible circular inhibition zone (Figure 3.4 C). The third class of interaction was one where there was an unclear zone (faint), indicating either partial inhibition of *C. albicans* growth or another form of interaction which influenced the growth properties of the ascomycete in the overlay agar (Figure 3.4 B). In the context of defining the prevalence of antimicrobial activity among Bcc isolates,

(A) No inhibition

(B) Faint inhibition zone

(C) Clear inhibition zone

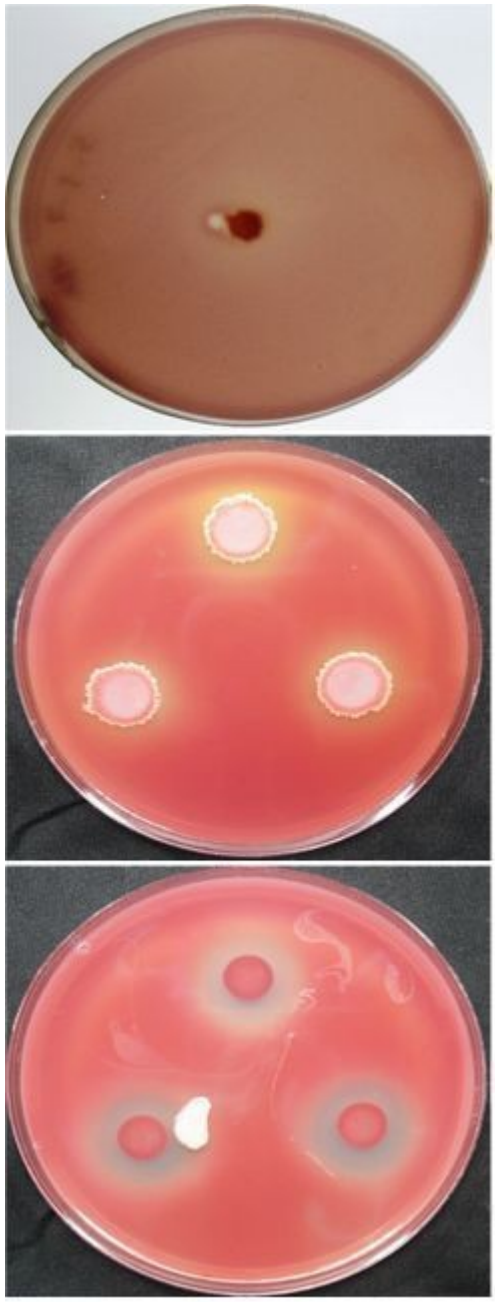


Figure 3.4 The effect of *Burkholderia* metabolites on *C. albicans*.

After incubation at 37°C for 24 h, the anti-candidal activity of Burkholderia was visible as; (A) no inhibition zone or (B) Faint inhibition zone or (C) clear inhibition zone on the red background of Candida cells which have grown and reduced the tetrazolium chloride metabolic indicator.

inhibition of *C. albicans* with a clear zone was considered as a positive effect and faint zones of clearing were considered as a negative result.

The ability of the 4 Bcc isolates to produce anti-candidal agents was not consistent and increased or decreased under growth different conditions (Table 3.2 and Figures 3.5). All four tested Bcc isolates failed to produce anti-candidal agents on MA of the six media tested. However, BSM-G, SA and PDA media (Figure 3.5) stimulated the four tested Bcc isolates to produce anti-candidal agents as follows.

B. ambifaria AMMD strain (Table 3.2) produced anti-candidal agents when grown on BSM-G and SA and the zone of inhibition was 29.6 ± 1.20 and 21.6 ± 0.88 mm respectively. The effects of BSM-G and SA media on anti-candidal production was highly significant ($P \leq 0.005$, F = 151.19). AMMD did not show anti-candidal activity when grown on MA, MHA, PDA and CMA.

B. ambifaria BCF isolates (Table 3.2) showed a lack of production of anti-candidal agents against all tested microorganisms when grown on MA, SA, PDA and CMA. However, anti-candidal agents were greatly enhanced when grown on BSM-G and MHA (59.6 ± 3.28 and 39.6 ± 1.20 mm respectively) and the effect of different media on anti-candidal production was highly significant ($P \leq 0.05$, F = 308.29).

B. ambifaria Kc311 isolates (Table 3.2) showed a lack of production of anti-candidal agents when grown on MA and MHA. Greatest inhibition by Kc311 was on CMA, BSM-G, SA and PDA (44.3 ± 1.20, 40.67 ± 0.67, 37.0 ± 1.0 and 29.0 ± 2.08 mm respectively)

Table 3.2 Effects of media type on production of anti-*Candida albicans* Sc5314 agents by four different Bcc isolates

Zone of clearing (mm) (mean ± S.E.M)							
		Media [a]					
		MA	SA	MHA	BSM-G	PDA	CMA
Bcc isolates [b]	AMMD	0.0	21.6 (0.88)	0.0	29.6 (1.20)	0.0	0.0
	BCF	0.0	0.0	39.6 (1.20)	59.6 (3.28)	0.0	0.0
	Kc311	0.0	37.0 (1.00)	0.0	40.67 (.67)	29.0 (2.08)	44.3 (1.20)
	JW13	0.0	8.7 (1.55)	0.0	50.0 (2.88)	41.7 (2.02)	0.0

[a] 2% Malt agar (MA), Sabouraud agar (SA), Muller Hinton agar (MHA), Basal salts media with glycerol (BSM_G), Potato dextrose agar (PDA) and Casein meat agar (CMA). [b] *B. ambifaria* AMMD, BCF & Kc311 and *B. vietnamiensis* JW13.

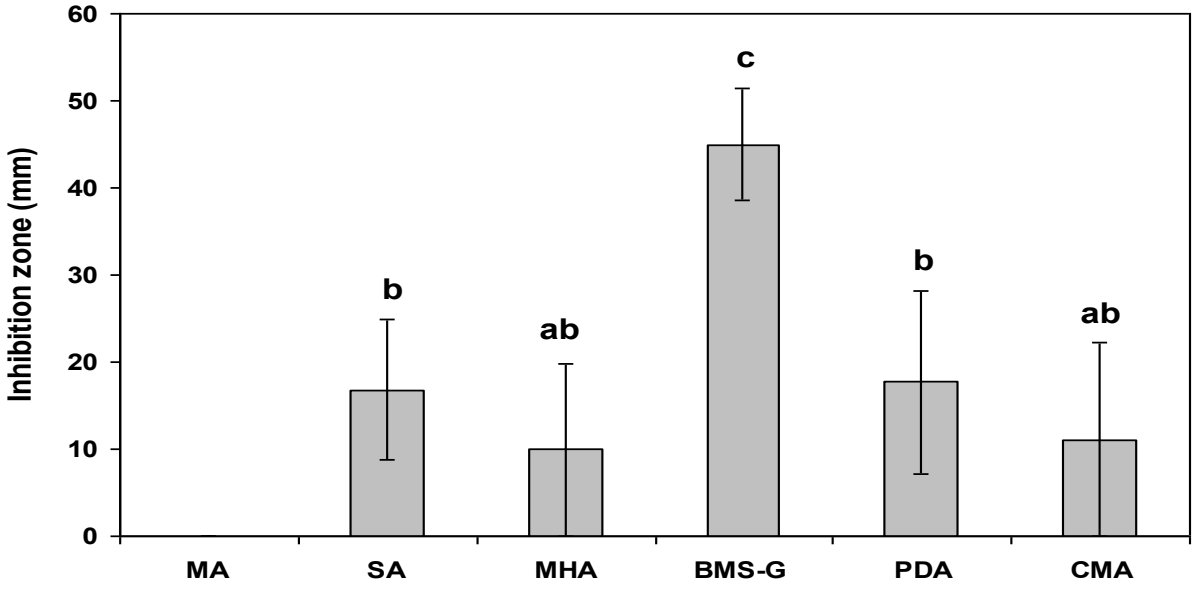


Figure 3.5 Effects of media type on production of anti-*Candida albicans* Sc 5314 agents by different *Burkholderia* strains at 30°C.

2% Malt agar (MA), Sabouraud agar (SA), Muller Hinton agar (MHA), Basal salts media with glycerol (BSM-G), Potato dextrose agar (PDA) and Casein meat agar (CAM). Different letters above columns indicate significant differences (ANOVA; $P \leq 0.05$). Data are presented as the means of three replicates with standard errors of the means.

and the effect of different media on antifungal production was significant ($P \leq 0.005$, F = 26.06).

B. vietnamiensis JW13 isolates did not produce anti-candidal activity when grown on MA, MHA and CMA. Anti-candidal activity was high when using BSM-G, PDA and SA (50.00 ± 2.88, 41.70 ± 2.02 and 8.7 ± 1.55 mm respectively) and the effect of different media on antifungal production was highly significant ($P \leq 0.001$, F = 103.73).

3.3.2 Interaction between *Burkholderia* and fungi

Examination of antibiotic production on 6 different media by the 4 Bcc isolates known to produce anti-candidal agents had shown that BSM-G and SA medium were suitable media for the production of these secondary metabolites. Therefore these media were selected to perform a systematic antifungal screen of 421 *Burkholderia* isolates recovered from clinical and environmental sources (Appendix A, Table A.1). The collection examined included 339 isolates from the *B. cepacia* complex (representing 11 formally Bcc named species and 4 novel groups that represent putatively novel species within the complex; Mahenthiralingam *et al*., 2008), 58 isolates from other *Burkholderia* species (39 from known *Burkholderia* species and 18 from isolates not identified to the species level), and 24 other non-*Burkholderia* bacteria isolates (Table 3.3). Antifungal activity against the ascomycete *C. albicans* Sc5314 (described as anti-candidal activity) and the basidiomycete *B. adusta* (described as anti-basidiomycete) was evaluated.

The raw data for antifungal activity (zone of inhibition for each isolate) is presented in Appendix A, Table A.1. Summative data for *B. cepacia* complex, *Burkholderia* species and non-*Burkholderia* species is presented in Table 3.3. General trends in the antifungal

activity observed were as follows. Growth of the bacteria and subsequently test for antifungal activity was most successful on SA medium. After growth on this medium 45.5% (192) isolates demonstrated anti-candidal and/or anti-basidiomycete activity (Table 3.3), and in 192 out of 421 cases, both activities were detected for the same isolate. Exceptions to this trend are described individually below. SA medium was also superior at enhancing the production of anti-candidal metabolites with 45.56% of isolates positive in comparison to BSM-G where 33% showed a zone of inhibition (this difference was, however, not significant; $P > 0.05$, Chi-Sq= 0.379). *B. stabilis* and *B. multivorans* failed to produce anti-fungal agents when grown on two different media: SA and BSM-G. However, *B. stabilis* was significantly successful (18%, $P \leq 0.001$, Chi-Sq= 13.8) in producing substances which suppressed *Candida* growth on PLA medium.

3.3.2.1 Interaction between *Burkholderia* and *C. albicans*

The interaction between the whole collection of *Burkholderia* and the reference strain *C. albicans* was carried out on two different media: SA and BSM-G. The results showed that the antagonistic Bcc species against *C. albicans* when using SA medium were Bcc6, *B. cepacia, B. cenocepacia* and *B. ambifaria* (100%, 86.6%, 77.1% and 56.8% respectively). However, *B. anthina* showed a poor ability to inhibit the growth of *C. albicans* (7.1%). Other species (*B. gladioli, B. glumae, B. oklahomensis* and *B. plantarii*) also produced very antagonistic agents (Table 3.3 and Figure 3.6).

The maximum zones of inhibition were recorded against *C. albicans* due to diffusible secondary metabolites of *Burkholderia* isolates when grown on BSM-G medium compared with SA medium and ranged between 82.3 – 7 mm and the maximum inhibition was

Table 3.3 Comparison of antifungal activity of *Burkholderia* and other bacterial species using two different media.

No	Tested isolates		Number and percentage of tested isolates	Number and percentage of active isolates producing antifungal agents		
				Anti-candidal		Anti-BK1
				BSM-G	SA	SA
1	***Burkholderia cepacia* complex (Bcc)**	***B. ambifaria***	101 (23.9%)	45 (44.1%)	58 (56.8%)	69 (67.6%)
2		***B. cenocepacia***	61 (14.5%)	12 (19.6%)	47 (77.1%)	47 (77.1%)
3		***B. cepacia***	30 (7.1%)	15 (50%)	26 (86.6%)	25 (83.3%)
4		***B. multivorans***	27 (6.4%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
5		***B. stabilis***	18 (4.2%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
6		***B. vietnamiensis***	18 (4.2%)	8 (44.4%)	4 (22.2%)	10 (55.5%)
7		***B. pyrrocinia***	16 (3.8%)	9 (56.2%)	9 (56.2%)	7 (43.7%)
8		***B. anthina***	14 (3.3%)	5 (35.7%)	1 (7.1%)	1 (7.1%)
9		***B. lata***	11 (2.36%)	4 (40%)	0 (0.0%)	1 (10%)
10		**Bcc 6**	10 (2.36%)	8 (80%)	10 (100%)	6 (60%)
11		***B. dolosa***	8 (1.9%)	0 (0.0%)	3 (37.5%)	4 (50%)
12		***B. contaminans***	6 (1.4%)	3 (50%)	5 (83.3%)	4 (66.6%)
13		**Bcc 5**	5 (1.18%)	4 (80%)	4 (80%)	0 (0.0%)
14		**Bcc 4**	3 (0.7%)	3 (100%)	2 (66.6%)	2 (66.6%)
15		**Bcc kc**	2 (0.47%)	1 (50%)	1 (50%)	2 (100%)
Total (Bcc)			339 (100%)	117 (29.3%)	170 (50.1%)	178 (52.5%)
16	**Other *Burkholderia****		58(13.7%)	22 (37.9%)	20 (34.5%)	13 (22.4%)
17	**Not *Burkholderia*** *		24 (5.7%)	1 (3.5%)	2 (7.1%)	1 (3.5%)
Total (All 1-17)			421 (100%)	140 (33%)	192 (45.5%)	192 (45.5%)

BSM-G; Basal salt medium supplemented with 0.4% glycerol, SA; Sabouraud agar, BKI; *B. adusta*

* The full name of species is listed in page 33 and table 2.2.

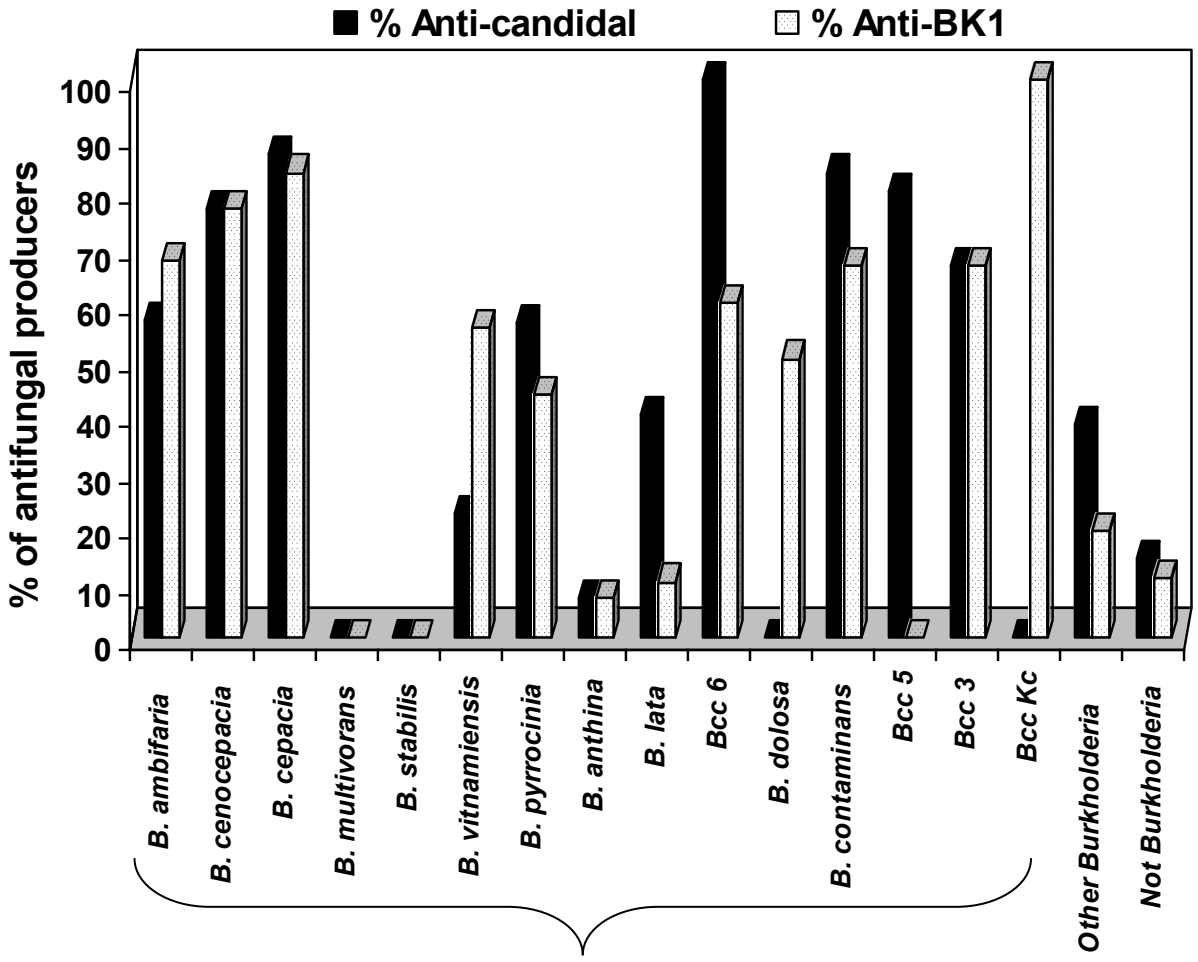


Figure 3.6 Effect of Sabouraud medium (SA) on the production of anti-*C. albicans* and anti-*B. adusta* BK1 agents.

339 isolates of *B. cepacia* complex, 58 isolates of other *Burkholderia* and 24 isolates of other bacteria (not *Burkholderia*) were tested for their antifungal activity.

observed by *B. plantarii*, *B. gladioli*, *B. phenazinum*, *B. ambifaria* and *B. vietnamiensis* (82.6, 82.2, 60.6, 53.3 and 52 mm respectively).

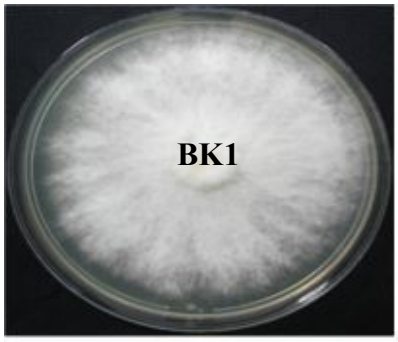
In general, the results showed that SA medium appeared to be the best at enhancing anti-candidal production by most tested bacteria with no significant differences between bacteria ($P > 0.05$, Chi-Sq = 0.379). Many of the Bcc isolates, for example, *B. cenocepacia*, *B. cepacia B. vietnamiensis* and *B. anthina,* showed highly significant differences in their production of anti-candidal agents when grown on two different media: SA and BSM-G ($P \leq 0.01$, Chi-Sq=34.03, $P \leq 0.05$, Chi-Sq= 9.83, $P \leq 0.01$, Chi-Sq= 7.4 and $P \leq 0.01$, Chi-Sq= 19.04 respectively). *B. ambifaria* also showed nearly the same results (56.86% and 4.11% respectively) with two different media: SA and BSM-G ($P \leq 0.2$, Chi-Sq = 1.60). In contrast, certain Bcc species preferentially produced anti-candidal agents when grown on BSM-G medium compared to SA medium: *B. vietnamiensis* (44.4%, 22.2%), *B. lata* (40%, 0.0%) and *B. anthina* (35.7%, 7.1%), ($P \leq 0.5$, Chi-Sq = 0.379; $P \leq 0.5$, Chi-Sq = 0.379 and $P \leq 0.5$, Chi-Sq = 0.379 respectively).

3.3.2.2 Interaction between *Burkholderia* and *B. adusta* BK1

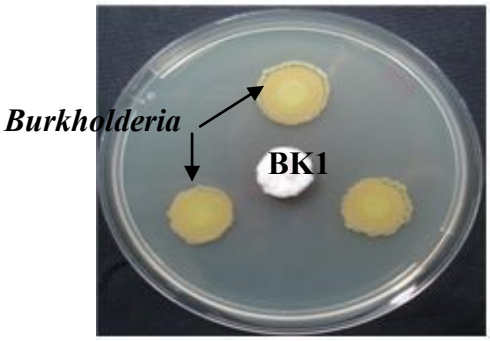
3.3.2.2.1 Using a contact antagonism assay

The interaction between a bacterial collection and model wood decay basidiomycete *B. adusta* was carried out on SA medium, using a contact antagonism assay (section 2.5.2.1). The effect of *Burkholderia* metabolites on *B. adusta* varied considerably from isolate to isolate. Three different categories of outcomes were observed. Firstly, a full inhibition of *B. adusta* growth (Figure 3.7 B) was defined when there was no growth at all of *B. adusta*. Secondly, deadlock (Figure 3.7 C) was defined when *Burkholderia* metabolites stopped the

A) BK1 control growth



B) Full inhibition



C) Deadlock

D) Over growth of BK1 and fan-like structure formation

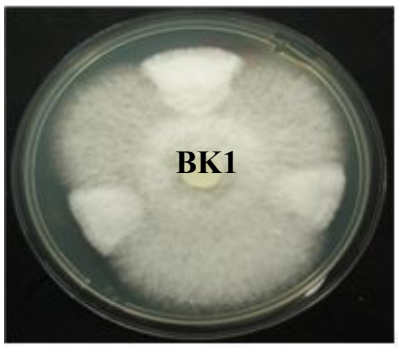


Figure 3.7 Outcomes of *Burkholderia* and *B. adusta* BK1 interactions.

After incubation at 25°C for 7 d the results were compared to *B. adusta* control plate without any *Burkholderia* (A). Anti- *B. adusta* activities were shown as: full inhibition (B) or deadlock (C) or over growth of BK1 and fan-like structure formation (D).

growth of *B. adusta* and finally, overgrowth of *B. adusta* on bacterial growth and fan-like structure formation (Figure 3.7 D) was defined when no fungal inhibition was observed. These findings illustrated a remarkable diversity of interactions between *Burkholderia* and basidiomycetes.

45.5% of tested bacteria (192 out of 421) were able to produce and release active metabolites which inhibited the growth of *B. adusta*. The most aggressive *Burkholderia* species against *B. adusta* (Table 3.3 and Figure 3.8) were *B. cepacia, B. cenocepacia*, *B. ambifaria* and Bcc6 (83.3%, 77.1%, 67.6% and 60% respectively). *B. gladioli*, *B. oklahomensis* and *B. plantarii* produced very active antifungal agents which inhibited the growth of *B. adusta*. The weakest species was *B. anthina* (7.1%). *B. stabilis* and *B. multivorans* did not inhibit the growth of *B. adusta*.

Many *Burkholderia* isolates were able to inhibit and/or change the morphology of *B. adusta* BK1 growth, there being clear variation in mycelial colour and hyphal densities compared to the control. For example *B. ambifaria* (Bcc0206, Bcc1080), *B. cepacia*, (Bcc0660, LMG 6964), *B. cenocepacia* (Bcc1014) and *B. gladioli* (Bcc0238, Bcc0507 and Bcc1623) were able to inhibit and deadlock the growth of *B. adusta* (Figure 3.9).

Burkholderia isolates, particularly *B. ambifaria* (Bcc0193), *B. terricola* LMG 20594 (Bcc0193), *B. fungorum* LMG 16225 (Bcc0770), *B. caribensis* LMG 20594 (Bcc0193) and other *Burkholderia* spp (Bcc0435 and Bcc0667) stimulated mycelia of *B. adusta* BK1 to produce fan-like structures (Figure 3.10) at mycelial margins compared to the control, and other species were able to grow over *Burkholderia* colonies and cover it.

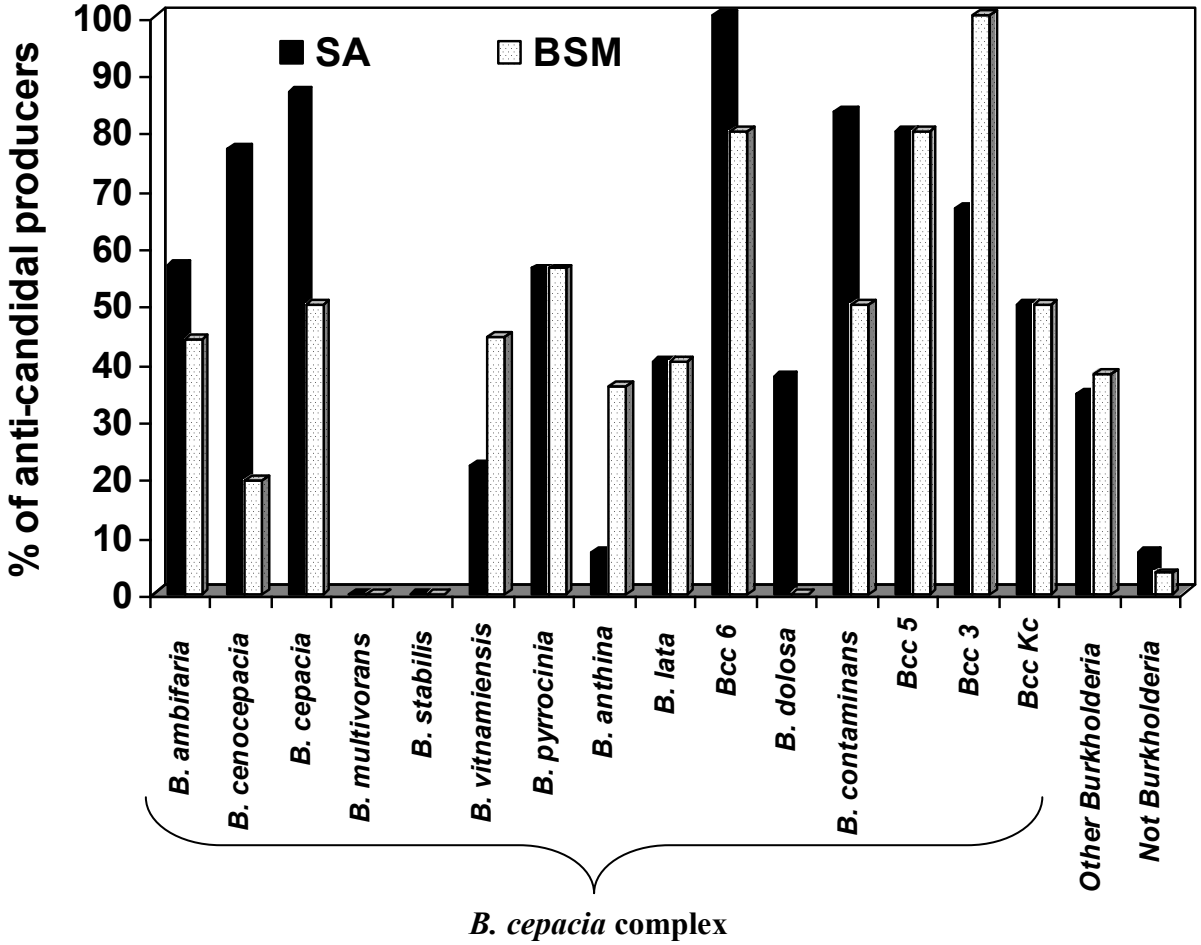


Figure 3.8 Effect of Basal Salt Medium (BSM) and Sabouraud medium (SA) on production of anti-*C. albicans* agents.

339 isolates of *B. cepacia* complex, 58 isolates of other *Burkholderia* and 24 isolates of other bacteria (not *Burkholderia*) were tested for their anti-candidal activity using two different media.

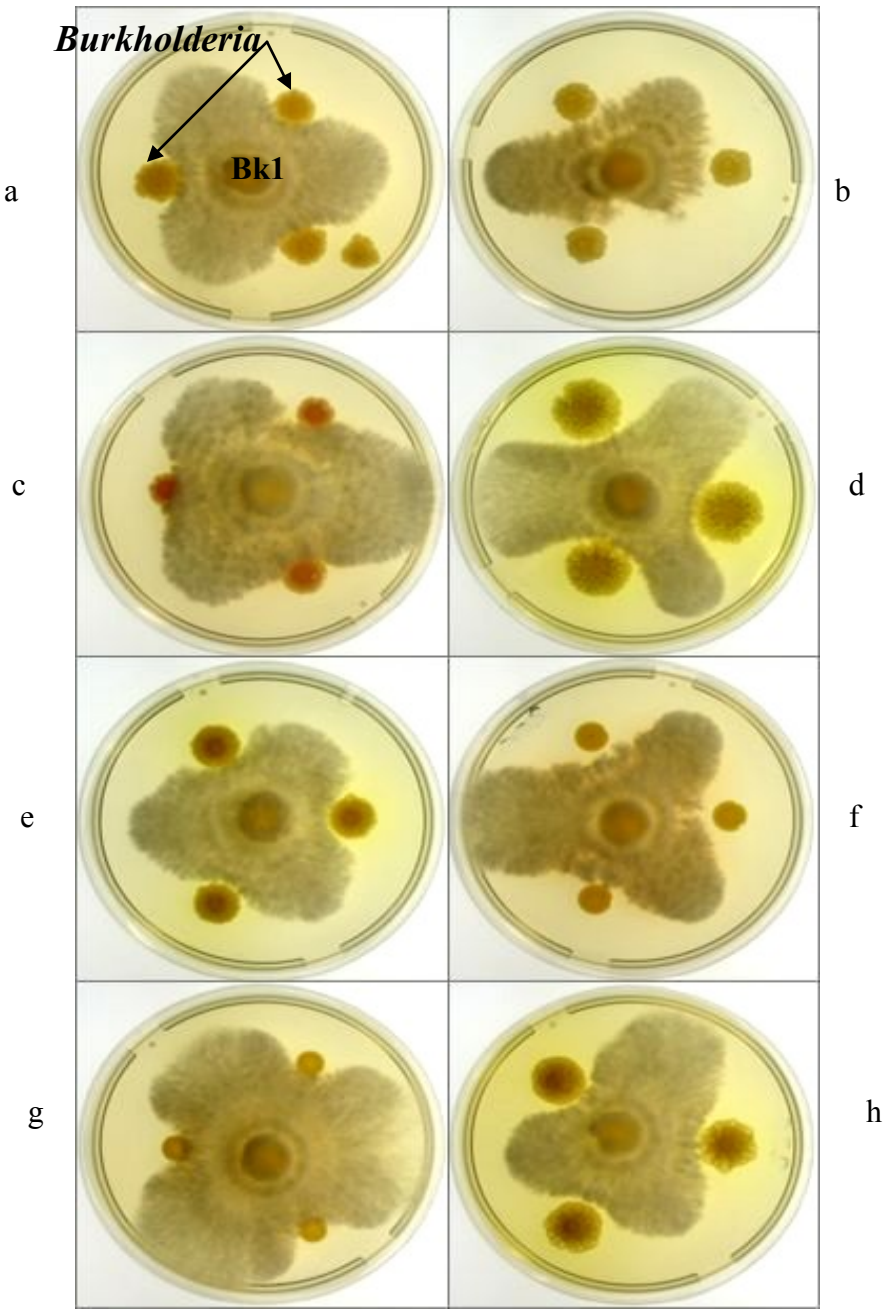


Figure 3.9 Deadlock of *B. adusta* BK1 growth as outcomes of the interaction with *Burkholderia* isolates.

(a) *B. gladioli* Bcc0507, **(b)** *B. cepacias* Bcc0660, LMG 6964, **(c)** *B. ambifaria* Bcc1080, **(d)** *B. gladioli* Bcc1620, **(e)** *B. gladioli* Bcc1623, **(f)** *B. ambifaria* Bcc0206, **(g)** *B. cenocepacia* Bcc1014 and **(h)** *B. gladioli* Bcc0238.

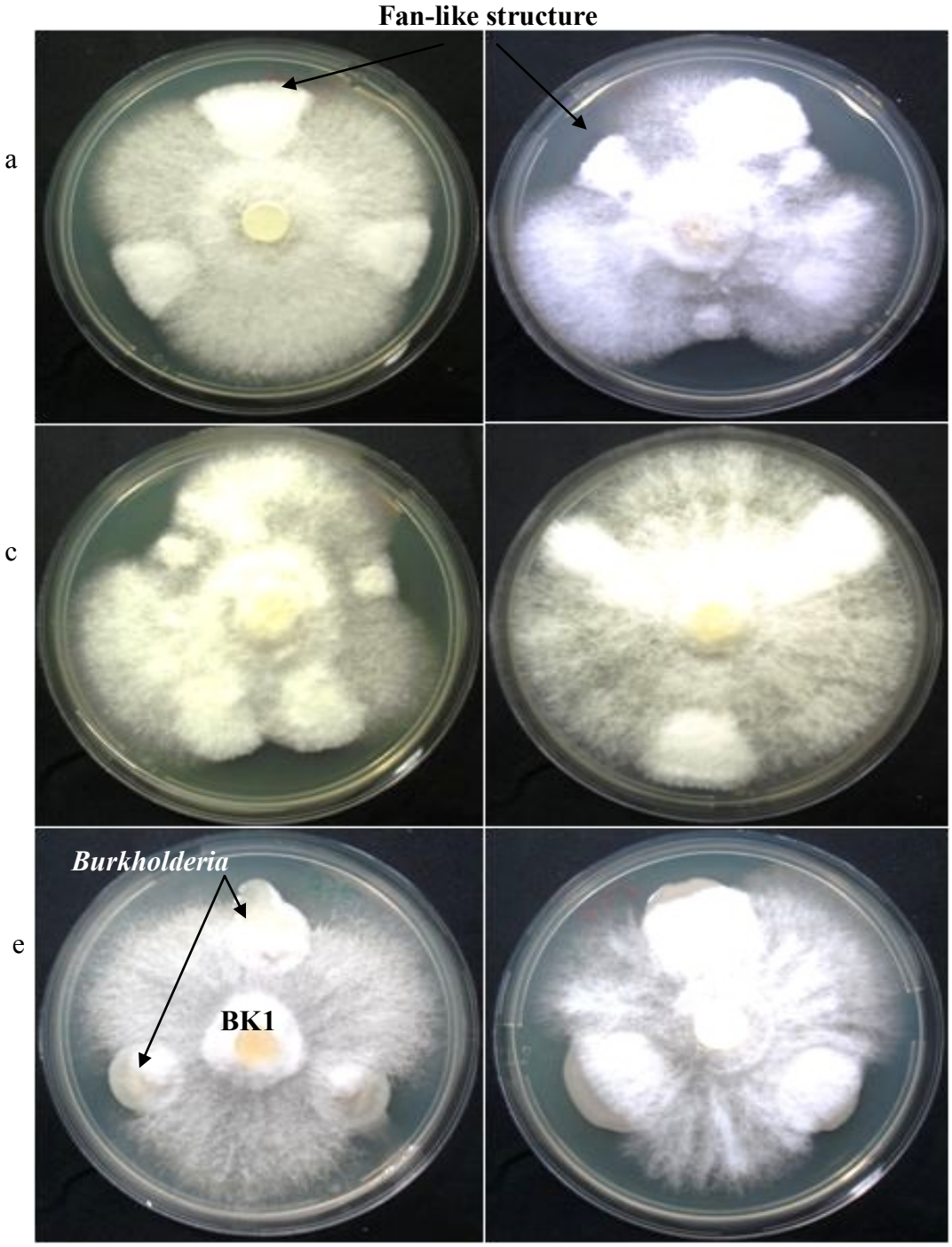


Figure 3.10 Overgrowth and fan-like structure formation of *B. adusta* BK1 as outcomes of the interaction with *Burkholderia* isolates.

(a) *B. terricola* Bcc1609, LMG20594, (**b)** *B. ambifaria* Bcc0193, (**c)** *B. fungorum* Bcc0770, LMG16225, (**d)** *Burkholderia* sp. Bcc0435, (**e)** *B. caribensis* Bcc0768, LMG18531 and (**f)** *Burkholderia* sp. Bcc0667.

B. adusta BK1 grew completely over many of *Burkholderia* species on SA medium during 7 d of incubation, and its mycelia were light or dense. *B. lata* LMG 22485 (Bcc0803) metabolites produced in SA medium decreased the growth of *B. adusta*, as the mycelium of *B. adusta* stopped just a few micrometers from *B. lata's* growth, with formation of a red pigment (Figure 3.11). In contrast, no pigmentation was observed when *B. lata* was exposed to other fungi or when grown alone.

3.3.2.2.2 Using basidiomycete overlay assay

Pouring an overlay of a test microorganism onto agar where a secondary metabolite producing microorganism has been grown forms a very simple assay to detect antimicrobial compounds. With microorganisms that grow planktonically as single cells or small clusters of cells, this overlay assay gives very reproducible and easy to observe results, as seen with the anti-*Candida* inhibition work. However, this classical type of overlay-inhibition assay has not been easy to apply to microorganisms which grown in a filamentous or clumping manner such as basidiomycete fungi. To develop an overlay assay suitable for application to the filamentous basidiomycetes, fragmentation of mycelia was evaluated as a means to produce an even inoculation and then subsequent fungal growth sufficient to observe the antifungal activity of secreted *Burkholderia* metabolites. Aliquots of homogenized mycelial inoculum were prepared using different size zirconium beads and fungal inocula grown under different conditions (see section 2.5.2.2). The best growth was observed when 0.25 mm zirconium beads were used to fragment 250 mg of fungal mycelia inoculum which had been grown on 2 ml SA medium for 2 days at 25°C under shaking conditions. Subsequent re-growth of 1 ml of fragmented fungal mycelia in the 25 ml soft

agar overlay medium was good and showed no bacterial contamination under experimental conditions.

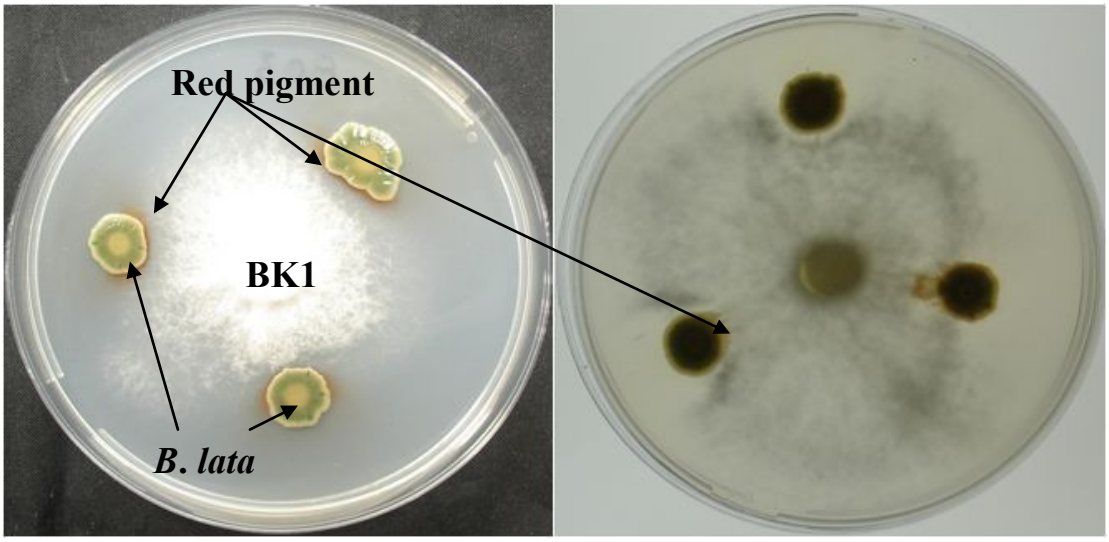


Figure 3.11 Production of red pigment from *B. lata* during interaction with *B. adusta* BK1.

B. lata suspension was spotted onto SA plate and incubated at 30°C for 3 d, and then the plate was centrally inoculated with *B. adusta* BK1. After incubation at 30°C for 5 d, the red pigment of *B. lata* was secreted.

Table 3.4 Zone of inhibition (mm ± SEM) of *B. adusta* and *T. versicolor* due to *B. ambifaria* AMMD, BCF, KW and MEX-5 diffusible metabolites using the basidiomycetes overlay assay

	AMMD	BCF	KW	MEX-5
B. adusta	51.75 ± 0.85	47.50 ± 1.19	37.75 ± 4.19	32.25 ± 1.11
T. versicolor	59.75 ± 0.63	58.00 ± 1.22	51.75 ± 1.18	41.00 ± 1.35

Five of the seven tested basidiomycetes were hard to optimize for overlay growth using the zirconium bead fragmentation assay. *H. fasciculare*, *P. impudicus* and *R. bicolor* did not grow reproducibly using this method. *P. velutina* produced weak growth that was barely visible. *S. gausapatum* produced a scattered variable growth that was unacceptable in the context of an even overlay assay. However, *B. adusta* BK1 and *T. versicolor* D2 showed an even layer of good growth covering the entire surface of SA plates within 3 and 4 d, respectively.

The ability to measure a zone of clearing using a basidiomycete-overlay assay enabled a more quantitative evaluation of antifungal activity compared to the contact-antagonism assay. It was also more comparable to the anti-candidal overlay assay since the *Burkholderia* strain could be left to secrete metabolites in the absence of the fungi, and then inactivated by chloroform vapour followed by assay of the anti-basidiomycete activity. The basidiomycete overlay assay was only applied to the 4 control *B. ambifaria* isolates since it had taken a while to develop. The inhibition zone due to *B. ambifaria* metabolites (Table 3.4 and Figure 3.12) ranged from 32.25 to 51.75 mm for *B. adusta* and 41 to 59.75 mm for *T. versicolor*.

The interaction of *B. ambifaria* AMMB, BCF, KW and Mex-5 against *B. adusta* BK1 and *T. versicolor* D2 was carried out on SA medium. The results showed that the antagonistic *Burkholderia* species against *B. adusta* BK1 were *B. ambifaria* AMMD and BCF (51 and 49 mm respectively). However, *B. ambifaria* Mex-5 and KW showed less ability to inhibit the growth of BK1 (40 and 32.25 mm respectively). The maximum zones of inhibition

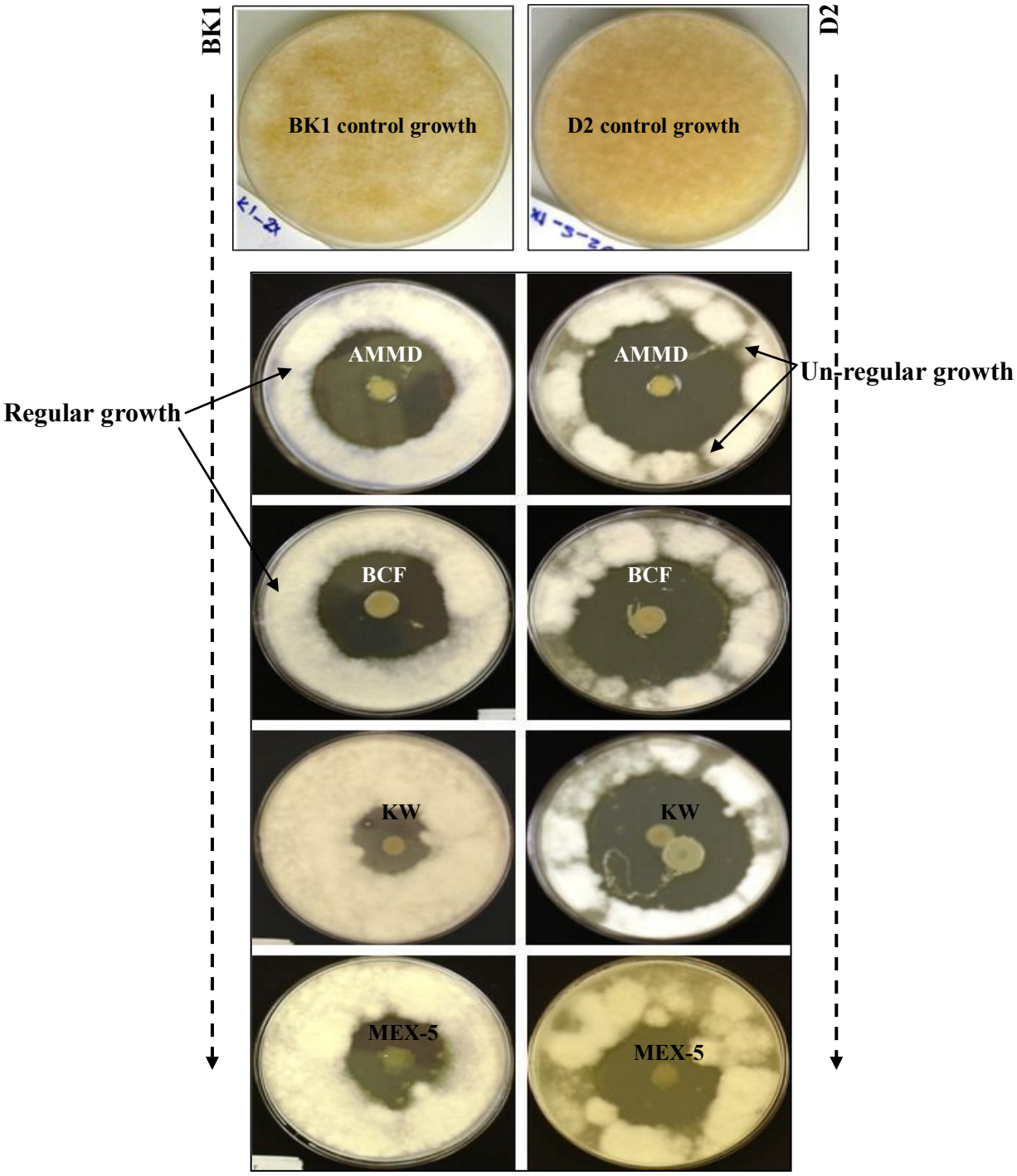


Figure 3.12 Zone of inhibition of *B. adusta* (BK1) and *T. versicolor* (D2) due to *B. ambifaria* AMMD, BCF, KW and MEX-5 metabolites using the overlay assay.

Burkholderia suspensions were spotted onto the center of SA plate and incubated at 30°C for 3 d. The plate was then overlaid with soft SA (1% Agar) seeded with fragmented BK1 or D2 inoculum. After incubation at 20°C for 3 d, the anti-basidiomycete activities of *Burkholderia* isolates were clearly visible as a clear zone of inhibition.

were recorded in interaction between *B. ambifaria* AMMD, KW and BCF and *T. versicolor* D2 on SA medium were 59.75, 58 and 56 mm respectively. Furthermore, *B. adusta* showed more regular growth after overlaying onto the secreted *Burkholderia* secondary metabolites than *T. versicolor*.

3.4 Discussion

Burkholderia (Tago*, et al.*, 2006) and basidiomycetes (A'Bear*, et al.*, 2012) are two of the dominant organisms in soil. Interactions between bacteria and fungi are affected by diffusible secondary metabolites including toxins, antibiotics and other metabolites or via bacteria-fungi interference or parasitism (Palumbo*, et al.*, 2007, Vial*, et al.*, 2008). To study the relationship between these organisms, growth conditions of both must be optimized. Bacterial-fungal interaction media should maintain both fungal and bacterial growth and support the production of secondary metabolites, especially those exhibiting antimicrobial activities, since microorganisms growing on different media can produce different metabolites and show different behavioural growth (Hwang*, et al.*, 2002, Li*, et al.*, 2007, Kadir*, et al.*, 2008). In this study, there was a need to search for suitable media which allow an equal opportunity for interaction between *Burkholderia* isolates and wood decay basidiomycetes, and thus explore new anti-fungal agents.

The ability of *Burkholderia* to use a broad spectrum of compounds as carbon sources is a known phenomenon and many of them are well known for the production of a variety of antifungal compounds (Parke & Gurian-Sherman, 2001). Studies have just begun to examine the antifungal ability of systematically identified Bcc species, with Mahenthiralingam *et al.*, (2011) specifically examining the anti-bacterial activity of 268

isolates. The work presented in this chapter has considerably expanded the latter study, looking at 339 Bcc and also 58 other *Burkholderia* species. In addition, the antagonism towards 10 representative wood decay basidiomycetes has been examined for the first time.

B. cepacia complex and other *Burkholderia* spp had varied capability to produce antifungal agents when grown on the same basal media. Four known antimicrobial producing Bcc bacteria were used to evaluate different growth media and our results showed that the best media to support production and activity of anti-candidal agents by *B. ambifaria* AMMD, BCF and Kc311 and *B. multivorans* JW13 were SA and BSM-G. However, rich media like TSA did not show activity. Murray and Woodward (2003) demonstrated a similar result with un-identified environmental bacteria as they confirmed that the best media to enhance antimicrobial production against wood decay fungi were SA and YPDA (Yeast Peptone Dextrose Agar) media. However, unlike this study they did not examine a minimal medium like BSM-G we have successfully used.

C. albicans has been used as a target microorganism to discover new antifungal substances. During its interaction with 421 bacterial isolates on BSM-G and SA media, the results revealed that SA medium was superior for screening, as 45.5% of isolates produced anti-candidal agents. The enhanced production may have occurred for a number of reasons. Firstly, this medium may contain precursors for anti-candidal metabolic activity, as Ismet *et al*., (2004) showed an increase in antifungal production when using peptone as a nitrogen source. Secondly, the anti-candidal activity might be due to the effect of the pH of the medium (pH 6), as many *Burkholderia* are isolated from acidic soils (Roitman*, et al.*,

1990). Similar enhancement of the production of antimicrobial agents at low pH has been seen in other studies (Roitman*, et al.*, 1990, El-Banna & Winkelmann, 1998). Overall, this may reflect the fact that *Burkholderia* bacteria are found at the highest densities in soils which are acidic, and hence their secondary metabolism and enzymic pathways are optimised towards this.

The production of anti-*Candida* and anti-*B. adusta* agents by Bcc isolates varied from one Bcc species to another, and also within the species. The differences in anti-fungal agent production reflect the considerable genetic variation seen between and within *Burkholderia* species. Of the basidiomycetes screened *B. adusta* showed the best ability to grow on all tested media and was therefore selected for use as an antagonistic model of wood decay fungi. The same overall number of isolates was active against *B. adusta* as were also active against *C. albicans* (Table 3.3). However, there were several groups of *B. cepacia* complex such as *B. ambifaria*, *B. vietnamiensis* and Bcc6 showed differential antagonism towards either the ascomycete or basidiomycetes. These similarities regarding the effects of *Burkholderia* isolates on both *C. albicans* and on *B. adusta* seem to be as a result of the ability of *Burkholderia* to produce many active substances with antifungal activity or due to the presence of active compound(s) that affect the growth of both *C. albicans* and *B. adusta*, or that different compounds might have the same mode of action. Li, *et al*., (2008) showed that the anti-candidal activity of some *Burkholderia* metabolites was as a result of losing the cell wall and altering the lipid and sterol contents of the cell.

The most active Bcc species were *B. ambifaria, B. cepacia* and *B. cenocepacia*; however, these species were all represented by 30 isolates or more (Table 3.3) and hence it could not

be ruled out that if more isolates in other species had been screened greater activity would have been seen. These Bcc species have been seen to be highly antifungal in previous studies (Vial*, et al.*, 2008). Quan, *et al*., (2006) showed that *B. cepacia* isolates produced strong anti-candidal agents and Li, *et al*., (2008) reported that strong anti-candidal activity of a CF66I compound was produced by *B. cepacia*. It is noteworthy that previous studies indicated that the most active *Burkholderia* producing antifungal compounds were *B. cepacia* and *B. ambifaria* AMMD, however, none of these studies examined large number of isolates.

B. stabilis and *B. multivorans* failed completely to produce anti-candidal and anti-*B. adusta* BK1 agents when grown on selected media SA and BSM-G. This might be due to the absence of those genes responsible for antifungal production, or due to the lack of specific ingredients in these media which are responsible for the activation of antifungal production. Using different media leads to the production of different metabolites and shows different behavioural growth (Hwang*, et al.*, 2002, Li*, et al.*, 2007). *B. multivorans* was unsuccessful in producing anti-candidal agents when grown on neo peptone and Lactose medium (PLA). Dikin, *et al*., (2007) demonstrated that PLA medium was able to induce antifungal production by a putative *B. multivorans* isolate (one isolate); this contrasts with the data from the present study, but we have screened many more isolates (27 isolates) than they did. Reflecting that *B. multivorans* was unable to produce anti-candidal agents under the tested conditions.

B. stabilis significantly (18 %, $P \leq 0.001$, Chi-Sq= 13.8) produced anti-candidal agents when grown on peptone lactose agar (PLA) medium. The importance of peptone in

enhancing the production of antifungal metabolites was previously reported (Schmidt, *et al.*, 2001). Furthermore, Dikin, *et al*., (2007) found that peptone and lactose were important supplements to enhance the production of antifungal metabolites by *B. cepacia* and *B. multivorans*. The effect of nutrients on antifungal production might be due to the effect of such compounds on the production and the efficiency of particular enzymes involved in this process or on the availability of important compounds needed to activate silent genes responsible for antifungal production.

Interactions between *Burkholderia* species and 10 different basidiomycetes on SA agar showed outcomes ranging from stimulation to inhibition. Some *Burkholderia* species on SA medium produced secondary antifungal metabolites which diffused to the surrounding agar and stopped or reduced the growth of tested fungi. In general *B. ambifaria*, *B. cepacia* and *B. cenocepacia* showed the strongest antagonistic activity against *B. adusta*. However, other *Burkholderia* isolates tended to stimulate the growth of *B. adusta* by producing stimulant metabolites that were presumably used as food or by using *Burkholderia* cells, after making contact (overgrowth) as an alternative source of protein and other important nutrients. Alternatively, the success of *B. adusta* might be as a result of defence mechanisms induced before direct contact.

The current results of *Burkholderia,* particularly *B. cepacia* complex (45% antifungal producers in Bcc collection) have provided a starting point for further experiments after the current study, to explore the ecology of *Burkholderia* and as an initial source for comparisons. It may also answer some essential questions about: the production conditions; the diversity of antifungal agents within *B. cepacia* complex, especially the

large collection of *B. ambifaria* and to determine the nature of these antifungal agents and the genetic basis of genes responsible for antifungal production.

Additional to antimicrobial activity of *Burkholderia*, other effects were seen for example, red pigment production and mycelial morphological changes. Red pigment produced by *B. lata* 383 was only observed during the interaction against *B. adusta.* This might be due to the effect of *B. adusta* metabolites which interfere with *B. lata* metabolism and reflect a highly species-specific interaction, or might be due to the quorum sensing (QS) effect. QS regulated the pigmentation and production of many other important metabolites, as Schmidt *et al.*, 2009 confirmed that the pigmentation in *B. lata* 383 was QS-controlled.

The fragmentation of mycelia of certain basidiomycetes was found to be a useful method to provide good growth that covered the Petri dishes in a short time (3 d) compared with the period of growth using the antagonism assay (7 d). This provided a regular distribution of fungal growth to determine the zone of inhibition due to diffusible bacterial metabolites. In general, the species which grew faster using fragmentation assays were *B. adusta* and *T. versicolor*. These species also showed high growth ability on different media (Table 3.1) after inoculation with a 5 mm agar plug of each fungus that was grown for 7 d on malt agar plates. However, the growth ability of basidiomycetes using the fragmentation assay was different from species to species and that might be caused by the nature of the fungal mycelium and the growth ability of each species. Alternatively, the method by which fragmentation is achieved might lead to damaged fungal mycelia which may in turn lead to a loss in or affect their ability to grow again.

3.5 Conclusions

Overall the following conclusions were reached:

- BSM-G and SA were the best media of those tested to screen for the production of antifungal agents by *Burkholderia*; with SA also highly suitable for subsequent antagonism assays with the basidiomycete fungi.
- *B. ambifaria*, *B. cepacia* and *B. cenocepacia* were the most antagonistic *Burkholderia* species against both *C. albicans* and *B. adusta*. However *B. multivorans* and *B. stabilis* appear to be species with a limited ability to produce antifungal agents.
- A basidiomycete overlay assay was developed and *B. adusta* BK1 and *T. versicolor* D2 were shown to be the most suitable species to screen in this way.
- The overall hypothesis for the chapter was rejected, in that not all Bcc species were found to be active in the secretion of antifungal agents under the conditions screened. However, the screen succeeded in identifying several potent anti-fungal *Burkholderia* isolates which can now be characterized.

CHAPTER FOUR

THE CHEMISTRAY AND ACTIVTY OF *Burkholderia* ANTIMICROBIAL AGENTS

4.1 Introduction

In comparison to bacteria such as *Streptomyces*, *Burkholderia* are relatively unexploited in the antibiotic discovery field. By screening for antifungal activity in a large collection of *Burkholderia* species and strains we have demonstrated that 45.5 % of isolates have antimicrobial activity (Chapter 3). To further explore the diversity of the anti-fungal activity within *Burkholderia*, methods for production, extraction and chemical analysis of the antimicrobial compounds need to be developed and evaluated. In addition, the activity of pure *Burkholderia* antibiotics on their target organism will need to be tested. New antibiotics and their producing organisms are in great demand at the present time, and if the characterization of *Burkholderia* can be brought forward they may provide a new resource for antibiotic discovery.

A profile of secondary metabolites of each organism consists of compounds produced under special growth conditions and includes enzymes, toxins, antibiotics and other unusual compounds. An advantage of using microorganisms to study secondary metabolites is that they can be grown under controlled conditions in the laboratory, which can be altered to mimic natural and un-natural conditions. For comparative purposes, microorganisms considered for chemical analysis must be grown on the same medium and under the same conditions of incubation to control the production of secondary metabolites. If the same antimicrobial extraction method is then applied, the metabolite profile obtained will primarily result from microbial diversity differences rather than due to the effect of environmental conditions (Sarker*, et al.*, 2006). To date no work has studied the diversity of anti-candidal compounds produced by a wide range of *Burkholderia*.

After the identification of antimicrobial activity in a pure culture, the extraction of

biologically active compounds is the next crucial step in the characterization of the antimicrobials. The development of suitable sample preparation techniques with significant advantages for the extraction and analysis of natural products plays an important role in providing a high quality and quantity of active natural products. Many studies have established various methods of extraction, characterization and identification of antimicrobial agents produced by *Burkholderia.* Different extraction techniques: maceration, liquid partition and adsorption (resin extraction) methods were used to extract biologically active constituents of *Burkholderia* metabolites in Mahenthiralingam lab, and were also conducted with a wide range of solvents with different polarities.

Thin layer chromatography (TLC) is a simple, rapid and economical procedure. The extract profile can be visualized, depending on the nature of these compounds. The degree of the fractionation depends on the nature of the compound mixtures under investigation, the type of adsorption layer and the power (polarity) of the mobile phase (Fritz & Schenk, 1987). The retention or retardation factor (R*f*) is commonly used to describe the chromatographic structure of any TLC profile. The R*f* value is the ratio of the distance the solvent travels to the distance the compound travels, and the center of each spot is the point at which the measurement is taken. R*f* values and other characteristics, such as the colour of metabolites make it possible to study complex mixtures qualitatively. TLC together with various visualization reagents offers the ability to obtain a nearly complete picture of microbial secondary metabolite patterns. TLC combined with a bioassay is a rapid and simple method that was developed in this current project to study the presence and the diversity of antifungal agents in *Burkholderia* secondary metabolites.

A number of methods can be used to begin to clarify the structure of active compounds including liquid-liquid chromatography (LC), mass spectrometry (MS), nuclear magnetic resonance (NMR), infrared spectrometry (IR) and ultra-violet (UV) absorption. A combination of LC-MS is used to study multi component mixtures for the presence of specific compounds. This method can be used to help identify known classes of compounds when searching for novel bioactive compounds. Molecular weight and UV absorbance data of bioactive natural products in a database allows rapid identification of compounds. These data can then be compared to a database of compounds that have already been tested. NMR is ultimately the method used to confirm the structural identity of a compound (Petr, 2008).

The general hypotheses for this chapter were: "*Burkholderia* bacteria produce more than one antimicrobial compound" and "TLC-Bioautography can be used to examine *Burkholderia* antimicrobial activity." The aims of this chapter were:

- To develop extraction techniques of active metabolites of *Burkholderia*.
- To optimize conditions of thin layer chromatography to enhance fractionation and visualization of *Burkholderia* active metabolites.
- To optimize the bioautography assay to detect active agents in each extract.
- To study the diversity of antimicrobial agents within *Burkholderia*.
- To purify and identify *Burkholderia* active metabolites.
- To determination the minimal inhibitory concentrations of crude and semi-purified *Burkholderia* active metabolites.

4.2 Materials and Methods

This present work was done to select the best conditions for the isolation, fractionation and identification of *Burkholderia* crude extracts and to determine the best conditions required to detect the active compounds of each extract. It also enabled detection of synergistic and antagonistic activity between compounds in each extract to show any antimicrobial activity effects.

4.2.1 Extraction techniques

Burkholderia antimicrobial metabolites were extracted using three methods of extraction (section 2.9): maceration, liquid-liquid and resin extraction. These fractions were then concentrated using a rotary evaporator at 30°C, and then kept at -20°C until use. The solid-phase extraction technique, resin extraction, was also used for the first time on *Burkholderia* anti-fungals in this study. Amberlite XAD16 resin, a neutral cross-linked polystyrene resin was used to extract secreted antibiotics from agar and broth cultures of *Burkholderia* (section 2.9). Extract was stored in microtubes at -20ºC until used.

4.2.2 Thin layer chromatography and bioautography assay (TLC)

As described in Section 2.10, TLC and different solvents with different polarities were used as a mobile phase to achieve the best fractionation of *Burkholderia* crude extracts. The developed TLC plates were observed under suitable visualizing reagents and the position of each spot was marked. The distance from the center of the spot to the point of the solvent front was then measured and the values of retention factor (R*f*) were obtained. A bioautography assay enabled rapid screening of natural extracts for antimicrobial activity. Many factors (section 2.10.1) were tested to detect the best conditions of

microbial growth on TLC plates such as: the affect of residual solvents, microbial inoculum size, pre-incubation and incubation conditions. The photos of developed TLC plates after the bioassay were taken under normal day light using a fixed digital camera (Sony, Cyber-shot, Japan).

4.2.3 Diversity of antimicrobial agents within *Burkholderia*

Eighteen *Burkholderia* isolates (Table 4.1) were chosen to investigate the diversity of anti-candidal activity within *Burkholderia*. The selection of a small panel of *Burkholderia* isolates was based on different criteria including superior activity against *C. albicans* and *B. adusta*. The diversity of anti-candidal activity within these *Burkholderia* extracts was investigated using a TLC-bioautography assay.

Four *Burkholderia* isolates *B. ambifaria* AMMD, BCF, KW and Mex-5 were chosen to further investigate the diversity of anti-basidiomycete activity within *Burkholderia.* These isolates were selected because they showed superior activity against *C. albicans*. The diversity of active metabolites produced by these *B. ambifaria* isolates against the following three basidiomycetes *B. adusta*, *T. versicolor* and *S. gausaptum* was investigated using a TLC-bioautography assay (section 2.10.2) and a basidiomycete agar overlay assay (section 2.5.2.2).

Six *Burkholderia* isolates *B. ambifaria* AMMD, BCF, KW and Mex-5, *B. vietnamiensis* Bcc1409 and *B. lata* Bcc0233 were selected to investigate the diversity of anti-bacterial activity against 10 different species of Gram-positive and Gram-negative bacteria (Table 4.2).

The TLC conditions used to separate pyrrolnitrin were adapted from previous studies as follows in order to compare the *B. ambifaria* active metabolites to this known antifungal. Pure pyrrolnitrin and eight of partially pure extracts of *B. ambifaria* AMMD were included in this comparison (section 4.3.4.1). Metabolite fractions were run in duplicate and compared side by side with pure pyrrolnitrin on the TLC plates under fixed conditions. The first run of these fractions and standard compounds were carried out on normal 0.25 silica gel TLC plates and treated with chloroform, ethyl acetate and formic acid 5:4:1 v/v as a solvent system (Burkhead*, et al.*, 1994). The second run was carried out on fluorescence 0.25 silica gel TLC plates and treated with chloroform and acetone 9:1v/v (Schmidt*, et al.*, 2009). One set of TLC plates was visualized under UV light at 366 nm and the other set was overlaid with *C. albicans*.

4.2.4 Purification and identification of *Burkholderia* active metabolites

Based on the results of the initial preliminary anti-candidal screening tests using TLC-bioautography assays*,* the crude extracts of *B. ambifaria* AMMD (Bcc0207), BCF (Bcc0203), KW (Bcc1256) and Mex-5 (Bcc1599) showed broad spectrums of activity against *C. albicans*, *B. adusta* and some bacteria. The attempt to isolate pure antimicrobials from *Burkholderia* metabolite extracts, three fractionation strategies were investigated to separate the *B. ambifaria* crude extracts into constituent active fractions, liquid fractionation (section 2.11.1), silica gel preparative thin layer chromatography (Section 2.11.2) and silica gel column chromatography (section 2.11.3). *Burkholderia* fractions were separately applied onto silica gel TLC plates to confirm purity and anti-candidal activity for further analysis. These extracts were subjected to further investigation using fractionation, isolation and identification by MS.

The information obtained from MS is important for the identification of certain types of organic compounds. Recently the advanced liquid chromatography conducted with mass spectrometry (LC-MS) has provided important information about product ions, and it is one of the most rapid and sensitive methods for detection and identification. Active *B. ambifaria* fractions of crude and semi-purified extracts from strains AMMD, BCF, KW and Mex-5 were sent to Prof. Gregory Challis's laboratory at Warwick University for further chemical analysis, performed by Dr. Lijiang Song.

4.2.5 *Burkholderia* antimicrobial activity

Crude and semi-pure antimicrobial extracts of *Burkholderia* were dried and then dissolved to set concentrations in Muller Hinton broth (MHB). A 96-well plastic micro-plate (Sterilin, UK) was used to perform a microdilution antimicrobial susceptibility assay following the Clinical Laboratory and Standards Institute (CSLI) guidelines (Sass*, et al.*, 2011). Serial dilutions of the antimicrobial extracts were made in the test set of wells. Standardised cultures of the susceptibility testing microorganisms (approximately 1 x 10^5 cfu ml^{-1}) were then added to each well. The plates were incubated at 37ºC for 24 h and the resulting microbial growth was quantified by measuring optical density at 600 nm using an automatic plate reader. The MIC value was identified as the lowest concentration of the antimicrobial extract which reduced the optical density to 80% of that of the control cultures (section 2.5.3).

4.3 Results

4.3.1 Extraction

Both maceration of growth agar and solid-phase resin extraction were able to isolate active *Burkholderia* metabolites. However, the extraction efficiency of the Amberlite XAD-16 resin followed by methanol elution proved to be the most robust method to extract the bioactive compounds from *Burkholderia.*

4.3.2 Optimization of TLC and bioautography assay

The solvents producing best fractionation of *Burkholderia* crude resin extracts were methanol, ethyl acetate, acetone and chloroform respectively. To improve the fractionation process of the crude resin extracts, these solvents were then mixed at different percentages to achieve suitable polarity. Ethyl acetate, methanol and water (EtOHc: MeOH: H_2O) at a 20:1:0.5 ratio was the best solvent system for separation of metabolites (Figures 4.1and 4.2). Methanol and water (8:2) was also capable of separating metabolites, but lacked the resolution of the EtOHc: MeOH: H_2O (Figure 4.3).

Most of the *Burkholderia* metabolites were not visible under day light before treatments with different chemical reagents. Long-wave length (366 nm) of UV radiation and iodine vapour (period of exposure 15 min) were the best means to visualize *Burkholderia* metabolites profiles on normal silica gel TLC glass plates.

Normal silica gel showed good separation of *Burkholderia* crude resin extracts (Figure 4.4). UV at 366 nm was easier to apply than iodine vapour development, and under UV light each single metabolite appeared as different colour. In contrast, florescence silica gel

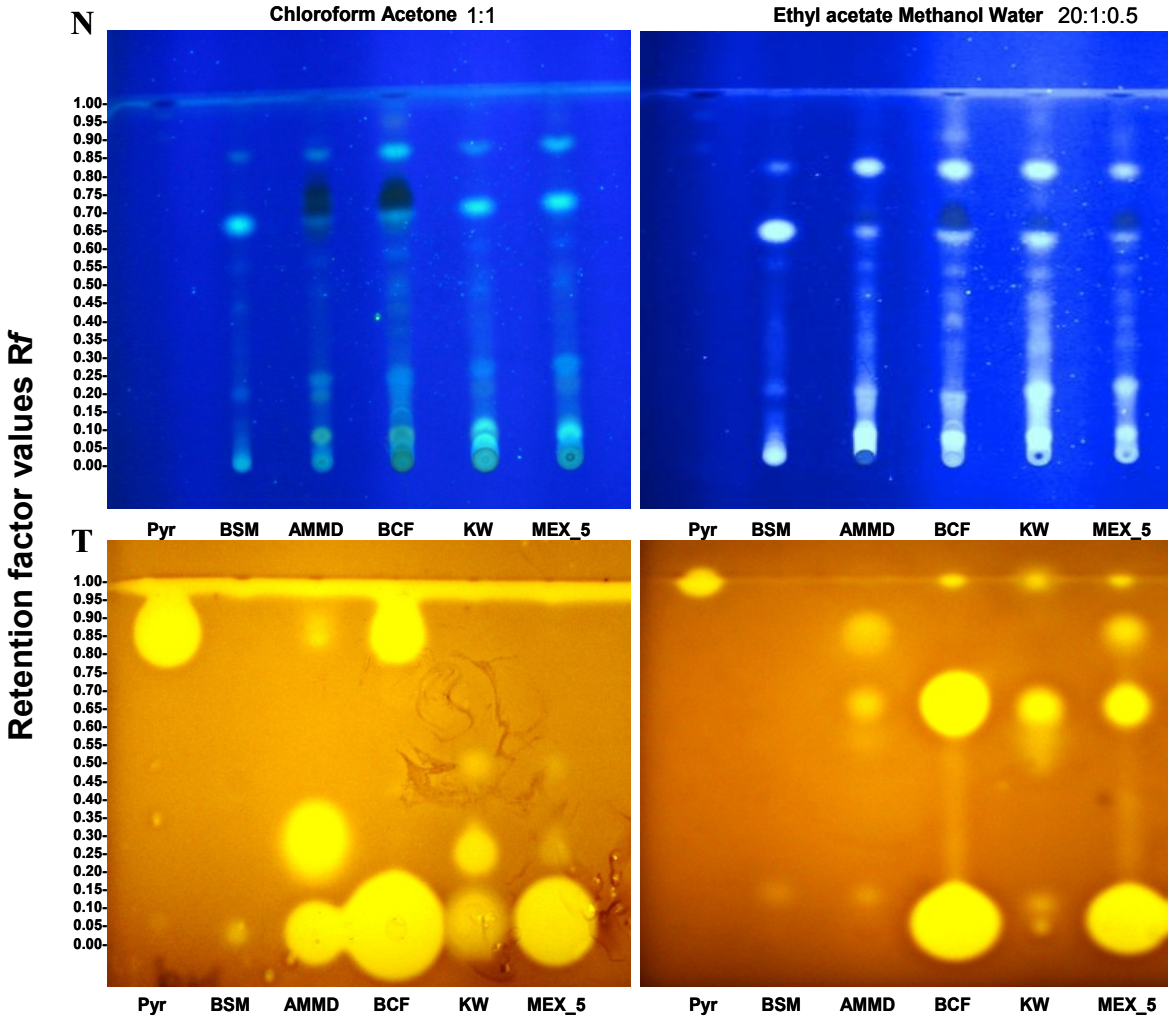


Figure 4.1 Fractionation of *Burkholderia* crude resin extracts using silica gel TLC.

Crude resin extracts of *B. ambifaria* isolates, pyrrolnitrin (Pyr) and Basal salts agar (BSM) were spotted onto TLC plates in duplicate then fractionated using ethyl acetate:methanol:water (20:1:0.5). One set of developed TLC plates was visualized using UV at 366 nm (Panel N).The other set was used for bioautography assay, the plate was overlaid with soft Isosensitest agar seeded with *C. albicans*, after incubation of plates at room temperature for 3 h and then 24 h at 37°C. The anti-candidal activity of different compounds is clearly visible as a clear zone on the red background of Candida cells which have grown and reduced the tetrazolium chloride metabolic indicator (Panel T). AMMD, *B. ambifaria* Bcc0207; BCF, *B. ambifaria* Bcc0250; KW, *B. ambifaria* Bcc1256; MEX-5, *B. ambifaria* Bcc1599.

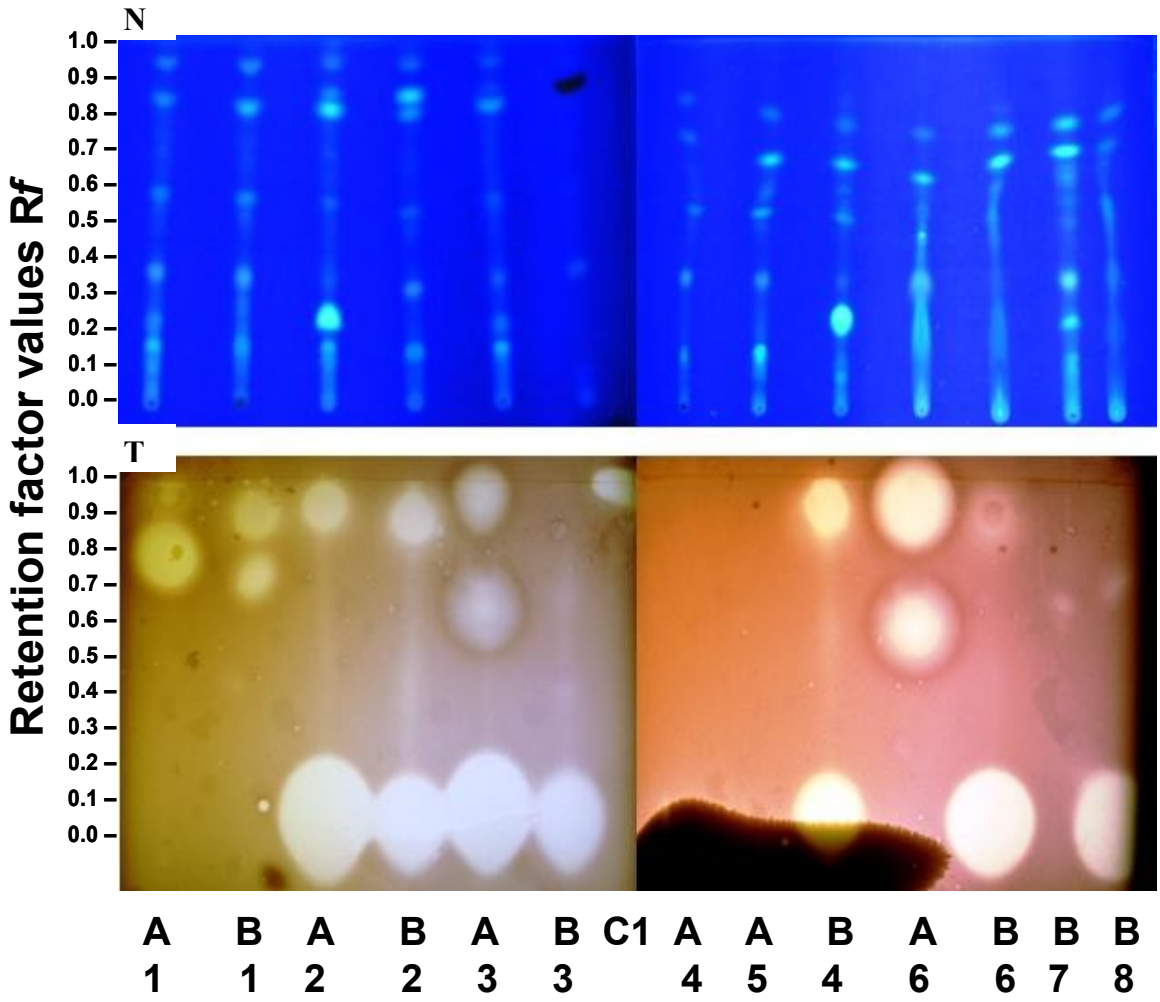


Figure 4.2 Fractionation of *B. ambifaria* crude resin extracts using silica gel TLC.

Crude resin extracts of 8 *B. ambifaria* isolates, pyrrolnitrin (Pyr) and Basal salts agar (BSM) were spotted onto TLC plates in duplicate then fractionated using ethyl acetate:methanol:water (20:1:0.5). One set of developed TLC plates was visualized using UV at 366 nm (Panel N).The other set was used for bioautography assay, the plate was overlaid with soft Isosensitest agar seeded with *C. albicans,* after incubation of plates at room temperature for 3 h and then 24 h at 37°C. The anti-candidal activity of different compounds is clearly visible as a clear zone on the red background of *Candida* cells which have grown and reduced the tetrazolium chloride metabolic indicator (Panel T). A, Sabouraud Agar; B, Basal Salt Agar; C1, Pyrrolnitrin; 1, *B. ambifaria* Bcc0207; 2, *B. ambifaria* Bcc410; 3, *B. ambifaria* Bcc1599; 4, *B. ambifaria* Bcc0267; 5, *B. ambifaria* Bcc0193; 6, *B. ambifaria* Bcc1265; 7, *B. ambifaria* Bcc0478; 8, *B. ambifaria* Bcc0203**.**

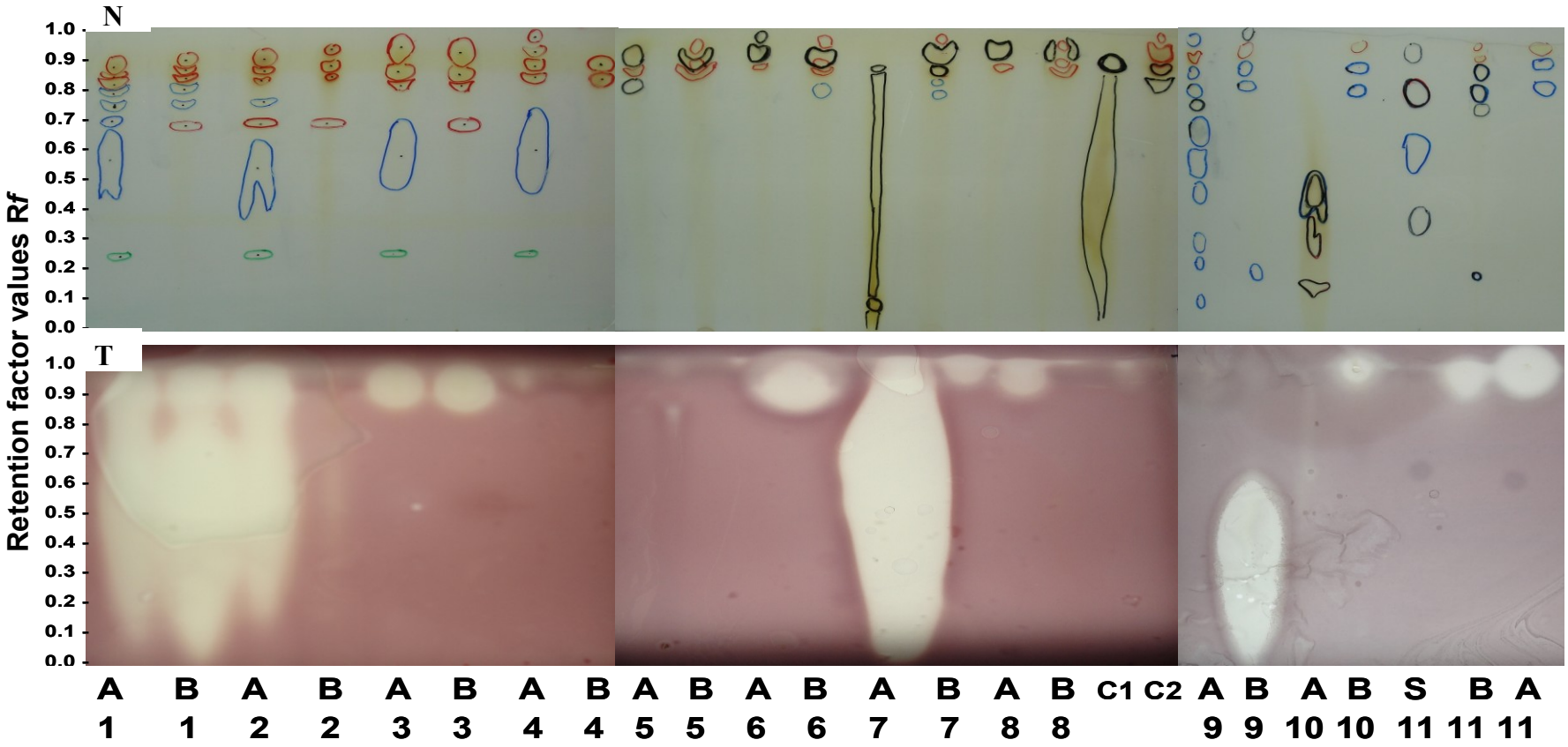


Figure 4.3 Fractionation of *Burkholderia* crude resin extracts using silica gel TLC and methanol: water (8:2).

Crude resin extracts of 10 *B. ambifaria* and *B. lata*, Sabouraud agar (C1) and Basal salts agar (C2) were spotted onto TLC plates in duplicate then fractionated in methanol:water (8:2). One set of developed TLC plates was visualized using iodine vapour (Panel N). The other set was used for bioautography assay, the plate was overlaid with soft Isosensitest agar seeded with *C. albicans*, after incubation of plates at room temperature for 3 h and then 24 h at 37°C. The anti-candidal activity of different compounds are clearly visible as a clear zone on the red background of Candida cells which have grown and reduced the tetrazolium chloride metabolic indicator (Panel T). 1, *B. ambifaria* Bcc0478; 2, *B. ambifaria* Bcc0267; 3, *B. ambifaria* Bcc0250; 4, *B. ambifaria* Bcc0193; 5, *B. lata* Bcc0193; 6, *B. ambifaria* Bcc1256; 7, *B. ambifaria* Bcc0410; 8, *B. ambifaria* Bcc0372; 9, *B. ambifaria* Bcc1241; 10, *B. ambifaria* Bcc1222; 11, *B. ambifaria* Bcc0207.

was not able to discriminate many of these metabolites using UV (Figure 4.4 N1). Furthermore, the visualising of *Burkholderia* metabolites separated on reversed phase C18 TLC plate was also un-successful (data not shown). The MeOH solvent showed extra capacity to extract many active metabolites compared with the acetone solvent which only extracted a fraction of the same compounds as MeOH.

Once the basic solvent conditions had been optimised, the microbial overlay system was then optimised. After development, it was found that the TLC plates needed to be aired for at least 15 min in a laminar flow cabinet before use. The inoculum size of the microorganisms for bioautography assay varied between the species used. The best inoculum size of *C. albicans* and gram-positive bacteria was 5 ml of 1 x 10^{-6} cfu per 100 ml of overlay agar. 1 ml of 1 x 10^{-6} cfu per 100 ml of overlay agar was optimal for gram-negative bacteria *B. multivorans*. Pre-incubation of overlaid TLC plates for at least 3 h at room temperature (20 ± 2°C) improved the detection of bioactivity, prior to the 24 h incubation at 37°C to allow the overlay organisms to grow. Many of the inhibition zones were associated with distinct compound spots and could be detected under the UV light as round areas of quenching.

4.3.3 Diversity of antimicrobial agents within *Burkholderia*

Eighteen *Burkholderia* isolates were chosen to investigate the diversity of anti-candidal activity within *Burkholderia*. This panel included 9 isolates of *B. ambifaria* and an isolate from each category of *B. cepacia* complex. *B. lata* (Bcc0308) was used as a positive control. Two *Burkholderia* isolates *B. ambifaria* Bcc0193 and Bcc0372 were included as negative controls. Extracts of the un-inoculated BSM and SA media were included as

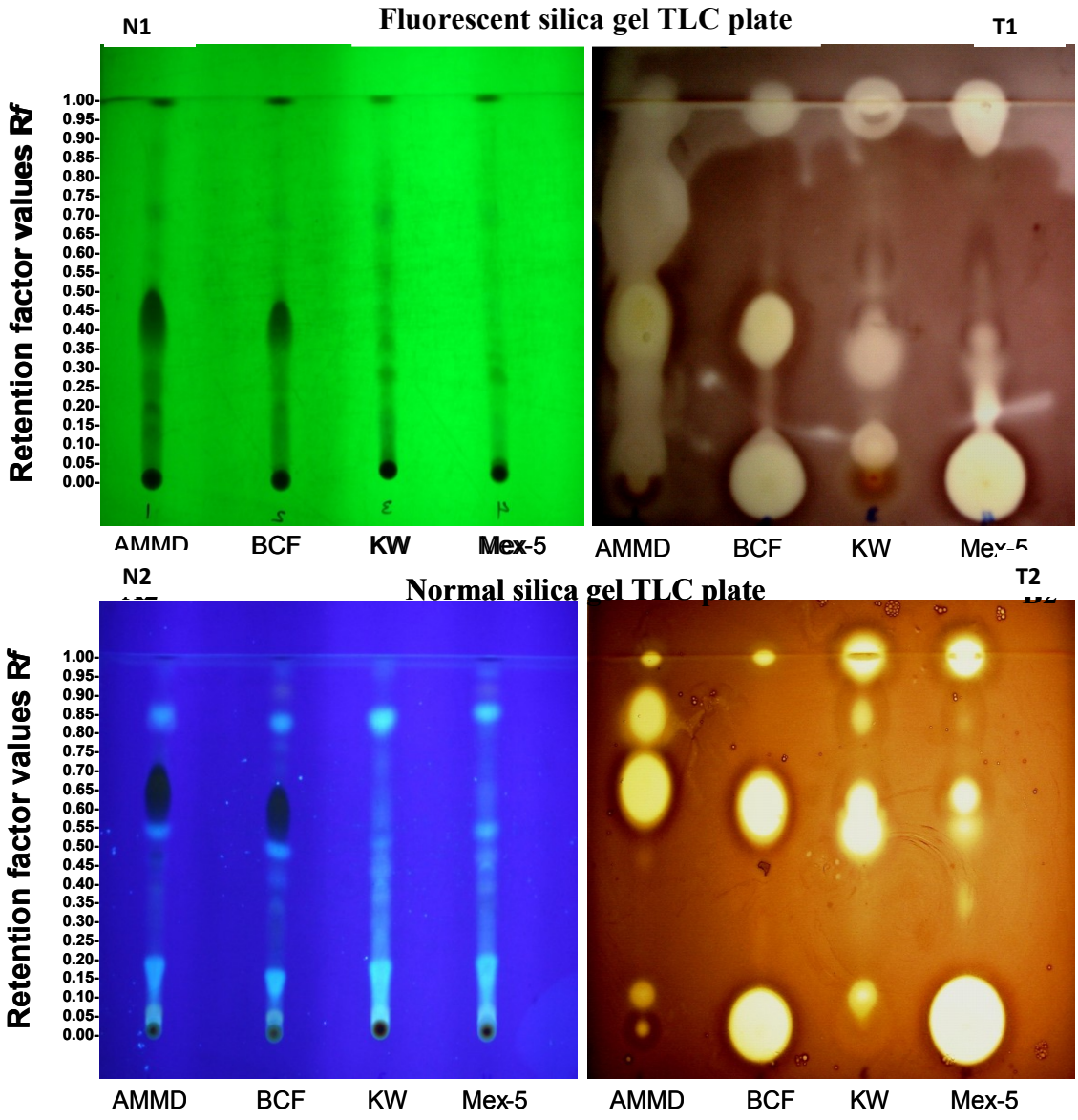


Figure 4.4 Fractionation of *Burkholderia* crude resin extracts using fluorescent and normal silica gel thin layer chromatography.

Crude resin extracts of *Burkholderia* were spotted onto fluorescent (N) and normal (T) TLC plates in duplicate then fractionated using ethyl acetate: methanol: water (20:1:0.5). One set of developed TLC plates was visualized using UV at 366 nm (Panel N1 & N2).The other set was used for bioautography assays (T1 & T2), the plate was overlaid with soft Isosensitest agar seeded with *C. albicans,* after incubation of plates at room temperature for 3 h and then 24 h at 37°C . The anti-candidal activities of different compounds are clearly visible as a clear zone on the red background of *Candida* cells which have grown and reduced the tetrazolium chloride metabolic indicator. AMMD, *B. ambifaria* Bcc0207; BCF, *B. ambifaria* Bcc0250; KW, *B. ambifaria* Bcc1256; MEX-5, *B. ambifaria* Bcc1599.

negative controls. Pure pyrrolnitrin (Sigma) and gladiolin were also included as positive controls (Table 4.1).

4.3.3.1 Diversity of anti-candidal agents

The TLC-bioautography profile obtained from control extracts made from un-inoculated BSM and SA media showed no antimicrobial activity (Table 4.1). Pure pyrrolnitrin migrated to the end of the TLC solvent front with R*f* value 0.97 ± 0.02 and showed anti-candidal activity with an inhibition zone of 17 mm (Appendix B, Figure B.1). From the screening of *Burkholderia* isolates for anti-candidal activity (section 3.3.2), several isolates produced very faint bioactivity zones in the candidal overlay; they were scored as inactive isolates because no clear zone of inhibition was seen. Crude resin extracts were prepared from representatives of these relatively inactive isolates to determine if the anti-candidal agents could be extracted and act more potently. For example, *B. ambifaria* Bcc0193 was previously shown to have no major inhibitory effect on candidal growth (section 3.3.2.1); the TLC-bioautography demonstrated that this isolate did produce metabolites with R*f*s 0.42, and 0.74 had a very limited "faint" affect on the candidal overlay (Table 4.1 and Appendix B, Figure B.1).

All sixteen active *Burkholderia* isolates examined were able to produce anti-candidal agents; they produced between 1 and 7 bioactive metabolites (Table 4.1). A total of 12 of the 18 *Burkholderia* isolates (66.6%) were able to produce active metabolites with similar R*f* of pure pyrrolnitrin (R*f* 0.97±0.02). The number and migration of active metabolites seen after growth on BSM-G and SA media were roughly the same (Table 4.1), with two major exceptions. *B. cepacia* Bcc0233 produced 5 active metabolites at R*f*s 0.0,

Table 4.1 TLC-bioautography assay of anti-candidal agents produced by 18 *B. cepacia* complex isolates.

Bcc isolates	Media	R_f of active metabolites (inhibition zone mm)									Active spots
		0.0	0.1	0.3	0.4	0.5	0.6	0.7	0.8	0.9	
B. ambifaria Bcc0267	BSM	0.02(2.5)	0.13 (20)	-	-	-	-	-	-	-	2
	SA	0.01(2)	0.14(27)	-	-	-	-	-	-	0.916(20)	3
B. ambifaria Bcc0410	BSM	0.01(16)	0.13(34)	-	-	-	-	-	-	0.916(20)	3
	SA	-	0.13(32)	-	-	-	-	-	-	0.90(21)	2
B. ambifaria Bcc0478	BSM	-	0.13 (36)	-	-	-	0.67(5F)	-	-	0.916(13)	2
	SA	NT									-
B. ambifaria Mex-5	BSM	0.01(10)	0.14(35)	0.35(14)	-	0.51(19)	0.65(30)	-	0.82(10)	0.95(17)	7
	SA	0.02(5)	0.12(30)	0.33(10)	-	0.50(22)	0.62(14)	-	-	0.96(11)	6
B. ambifaria Bcc0193	BSM	-	-	-	0.42(3f)	-	-	-	-	-	0
	SA	-	-	-	-	-	-	0.74(3f)	-	-	0
B. ambifaria Bcc0372	BSM	-	-	-	-	-	-	-	0.86(3f)	-	0
	SA	-	-	-	-	-	-	-	0.86(2f)	-	0
B. ambifaria KW	BSM	0.09(25)	-	-	-	0.5(16)	0.63(20)	-	0.82(10)	0.95 (22)	5
	SA	-	0.16(14)	-	-	0.52(19)	0.67(5f)	-	0.85(8)	-	4
B. ambifaria AMMD	BSM	0.01(6)	0.10(5)	-	0.45(17)	-	0.67(20)	-	0.81(21)	0.97(8)	6
	SA	0.02(5)	0.11(4)	-	0.42(10)	-	0.64(11)	0.77(14)	-	0.93(20)	6
B. ambifaria BCF	BSM	-	0.122(30)	-	-	-	0.64(23)	-	-	0.97(8)	3
	SA	0.08 (23)	-	-	-	-	0.61(17)	-	-	0.93(20)	3
B. vietnamiensis Bcc1409	BSM	-	-	-	-	-	0.65(14)	0.73(5)	-	0.92(26)	3
	SA	NT									-
B. contaminans Bcc1315	BSM	-	-	-	-	-	-	-	-	0.906(18)	1
	SA	0(1)	-	-	-	-	-	-	-	0.906(18), 0.98(8)	3
B. anthina Bcc1350	BSM	-	0.107(20)	-	-	-	-	-	-	0.903(14)	2
	SA	-	0.107(15)	-	-	-	-	-	-	0.9(18)	2
B. cepacia Bcc0233	BSM	-	-	-	-	-	-	-	-	0.98(f)	0
	SA	0(2)	-	0.35(4)	0.46(2)	-	-	0.75(4)	-	0.98(22)	5
B. cepacia Bcc0004	BSM	NT									
	SA	-	-	-	0.48(f)	0.58(f)	-	0.786(f)	-	0.978(f)	0
B. cenocepacia Bcc0019	BSM	-	-	-	-	-	-	-	-	0.90(13)	0
	SA	-	-	-	-	-	-	-	-	-	1
B. cenocepacia Bcc0165	BSM	-	-	-	-	-	-	-	-	0.90(11)	1
	SA	-	-	-	-	-	-	-	-	0.92(7)	1
B. lata Bcc0803	BSM	-	-	-	-	-	-	0.73(3)	-	-	1
	SA	-	-	-	0.48(4)	-	-	0.72(2)	-	-	2
B. pyrrocinia Bcc0171	BSM	-	-	-	0.48(2)	-	-	-	-	-	1
	SA	-	-	-	0.49(2)	0.58(2)	-	0.72(2)	-	-	3
Pyrrolnitrin (positive control)		-	-	-	-	-	-	-	-	0.978(17)	1
BSM (negative control)		-	-	-	-	-	-	-	-	-	0
SA (negative control)		-	-	-	-	-	-	-	-	-	0

R*f*, Retention factor; values in brackets show the mean of inhibition zone in mm; f, faint effect; NT, not tested; BSM, basal salt medium; SA, Sabouraud medium.

0.35, 0.46, 0.75 and 0.98 when grown on BSM medium and produced only 1 compound with a faint affect at R*f* 0.9 when grown on SA (Table 4.1). *B. cenocepacia* Bcc0019 produced only one anti-candidal compound when grown on BSM-G medium at R*f* 0.98 and it did not produce any active agents when grown on SA medium (Table 4.1 and Appendix B, Figure B.1: 13A, 13B).

B. ambifaria Mex-5, AMMD and BCF were the most active isolates when both the size of the inhibition zones and the number of active anti-candidal metabolites were considered (Table 4.2). *B. ambifaria* Mex-5 produced at least 7 active metabolites when grown on BSM-G medium at R*f*s 0.01, 0.14, 0.35, 0.51, 0.65, 0.82 and 0.95 with different inhibition zones ranging from 10 mm to 35 mm. A slight difference in the production of anti-candidal agents was observed between BSM-G and SA media, as one of anti-candidal agents with R*f* 0.82 was absent. *C. parapsilosis* showed nearly the same sensitivity against *Burkholderia* metabolites as *C. albicans* (Table 4.1 and Appendix B, Figure B.2).

4.3.3.2 Diversity of anti-basidiomycetes agents

As had been done with the anti-candidal assays, the *Burkholderia* crude resin extracts were fractionated on silica gel TLC plates using ethyl acetate, methanol and water (20:1:0.5) prior to analysis of anti-basidiomycete activity (Figure 4.5, B). The subsequent bioautography testing involved using the novel fragmented mycelial overlay assay for basidiomycetes *B. ad*usta, *T. versicolor* and *S. gausapatum*, that had been successfully developed previously (section 2.5.2.2).

According to the TLC-bioautography profile (Figure 4.5, A and Table 2), the results

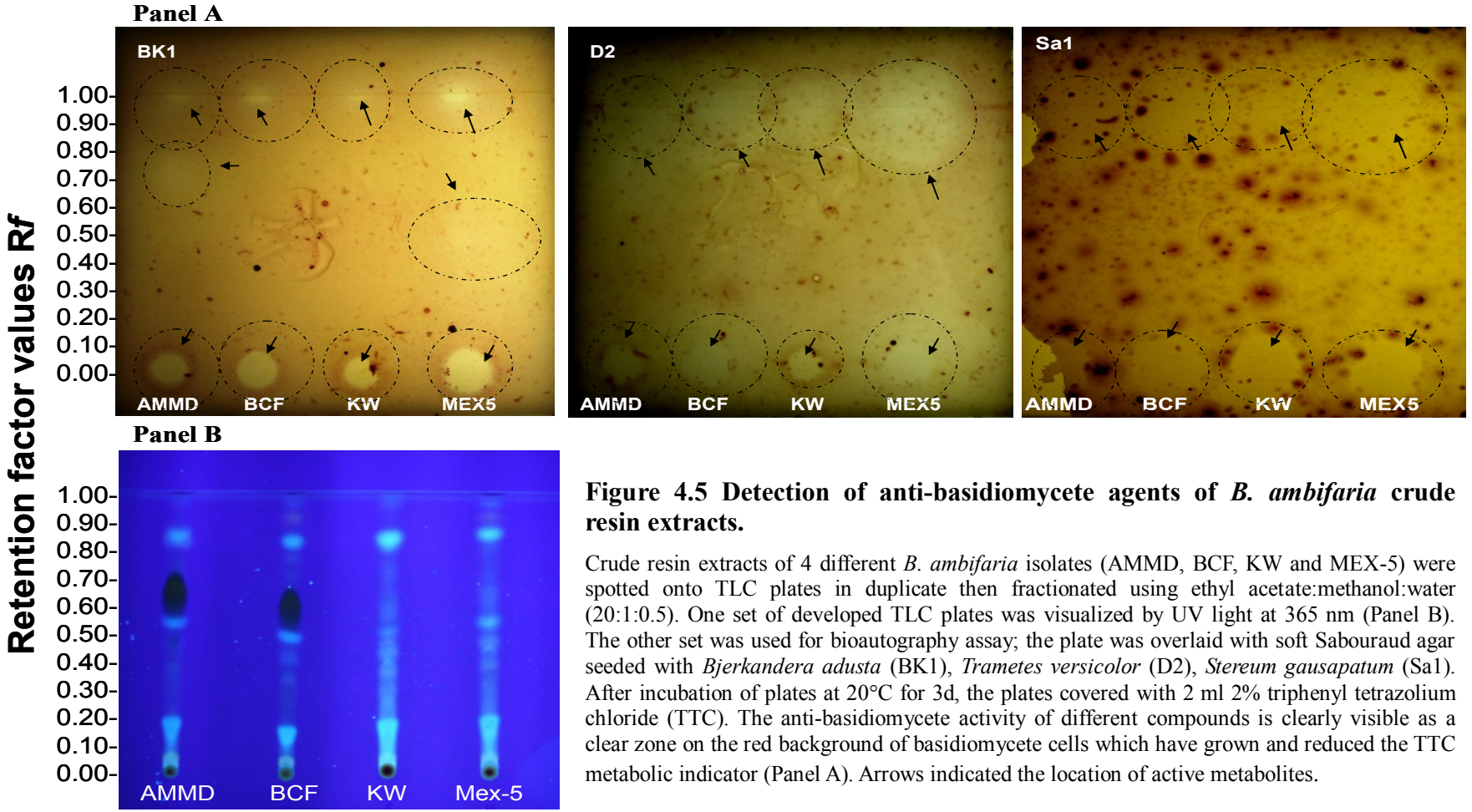


Figure 4.5 Detection of anti-basidiomycete agents of *B. ambifaria* crude resin extracts.

Crude resin extracts of 4 different *B. ambifaria* isolates (AMMD, BCF, KW and MEX-5) were spotted onto TLC plates in duplicate then fractionated using ethyl acetate:methanol:water (20:1:0.5). One set of developed TLC plates was visualized by UV light at 365 nm (Panel B). The other set was used for bioautography assay; the plate was overlaid with soft Sabouraud agar seeded with *Bjerkandera adusta* (BK1), *Trametes versicolor* (D2), *Stereum gausapatum* (Sa1). After incubation of plates at 20°C for 3d, the plates covered with 2 ml 2% triphenyl tetrazolium chloride (TTC). The anti-basidiomycete activity of different compounds is clearly visible as a clear zone on the red background of basidiomycete cells which have grown and reduced the TTC metabolic indicator (Panel A). Arrows indicated the location of active metabolites.

showed that all *B. ambifaria* isolates were able to produce anti-basidiomycete metabolites which inhibited the growth of the 3 tested fungi. *B. ambifaria* AMMD, BCF, KW and Mex-5 produced 2 active anti-*T. versicolor* and anti-*S. gausaptum* metabolites at R*f*s 0.02 and 0.98 (Figure 4.5, panel A: D2 and Sa1). *B. ambifaria* AMMD and Mex-5 produced 3 active anti-*B. adusta* metabolites at R*f*s 0.02, 0.70 and 0.98. In addition, *B. ambifaria* BCF and KW produced 2 active anti-*B. adusta* metabolites at R*f*s 0.02 and 0.98 (Figure 4.5, panel A; BK1).

4.3.3.3 Diversity of anti-bacterial agents

Pure pyrrolnitrin migrated up to the end of the TLC solvent front with R*f* values 0.97 ± 0.02, and showed anti-bacterial activity against all tested bacteria, except *S. maltophilia* LGM958 and *A. baumannii* (Table 4.2 and Appendix B; Figure B.2). All tested *Burkholderia* produced active antibacterial metabolites with R*f* values 0.97 ± 0.03 which corresponded to that of pure pyrrolnitrin (Table 4.2).

B. ambifaria AMMD, BCF, KW and Mex-5 produced a wide range of active antibacterial metabolites. They produced up to six active metabolites which exhibited greater effect against Gram positive than Gram negative bacteria (Figure 4.6). The profile of active anti-Gram positive compounds largely matched those of the anti-candidal metabolites (Figure 4.1) for the *Burkholderia* screened.

Active metabolites of *B. ambifaria* AMMD (R*f* 0.45 ± 0.05) with yellow colour, inhibited all Gram-positive and Gram-negative bacteria tested. However, *B. ambifaria* BCF metabolites (R*f* 0.01 ± 0.01) with a brown colour were most active against Gram-positive

bacteria (Figure 4.6 for *S. aureus*; Table 4.2), but also showed good activity against *P. fluorescens* (Table 4.2 and Figure 4.6) and weaker activity on *E. coli* (Table 4.2 and Appendix B, Figure B.2). Other BCF metabolites migrating at R*f* 0.45 ± 0.05 with a yellow colour also showed good anti-Gram positive activity, and some activity against *P. fluorescens* (Table 4.2 and Figure 4.6).

B. ambifaria KW Metabolites (R*f* 0.41 ± 0.01, 0.70 ± 0.01, 84 ± 0.02 and 0.99 ± 0.01) were able to inhibit the growth of *E. faecalis* ATCC51299 (Figure 4.6, A) and *P. fluorescens* LMG1974 (Figure 4.6, D). Furthermore, other KW metabolites (R*f* 0.69 ± 0.02, 0.83 ± 0.01 and 0.99 ± 0.01) inhibited the growth of all Gram-positive bacteria (Table 4.2).

In *B. ambifaria* Mex-5 only one metabolite (R*f* 0.01 ± 0.01) was able to inhibit the growth of all tested bacteria (Figure 4.6 and Appendix B, Figure B2) except Gram-negative *S. maltophilia* LMG958 (Appendix B, Figure B2) and *A. baumannii* (Figure 4.6, C) and the metabolites detected at R*f*s 0.83 ± 0.02 and 0.98 ± 0.02 inhibited the growth of all tested bacteria except *S. maltophilia* LGM958, *A. baumannii, E. coli* NCTC1224 and *B. cenocepacia* LMG16656 (Table 4.2, Figure 4.6 and Appendix B, Figure B2).

B. vietnamiensis Bcc1409 produced a range of antibacterial metabolites which mainly affected the growth of the Gram-positive bacteria (Table 4.2). In contrast, *B. lata* Bcc233 metabolites (R*f*s 0.02, 0.08, 0.16, 0.42, 0.53, 0.66, 0.81 and 0.99), inhibited the growth of Gram-positive *S. aureus* strains, except NCTC12981 (Appendix B, Figure B2). However, this extract was inactive against other gram negative bacteria (Appendix B, Figure B2).

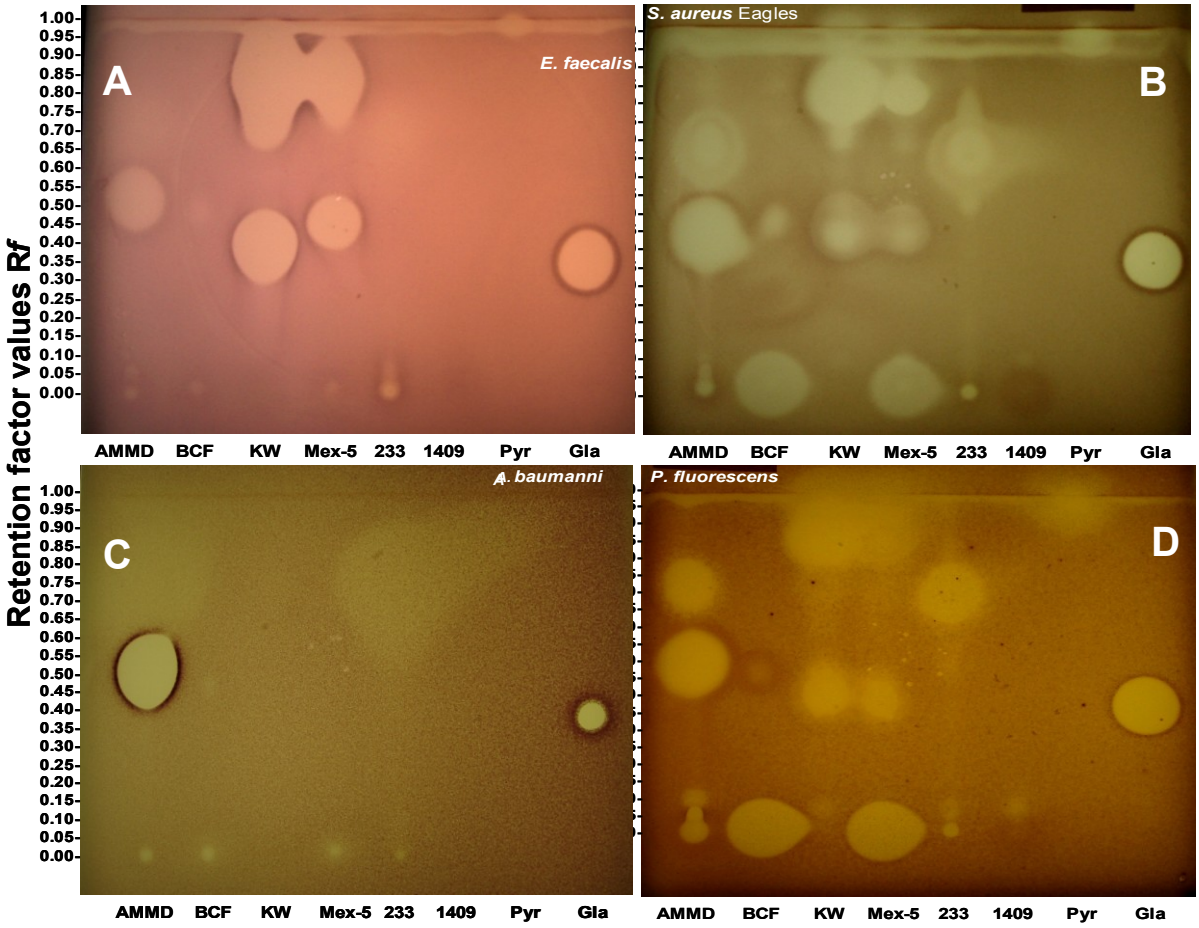


Figure 4.6 Detection of antibacterial agents of *Burkholderia* crud resin extracts.

Crude resin extracts of 6 *Burkholderia*, pyrrolnitrin and pure gladiolin were spotted onto TLC plates in duplicate then fractionated using ethylacetate:methanol:water (20:1:0.5). One set of developed TLC plates was visualized using UV at 366 nm. The other set was used for bioautography assays, the plate was overlaid with soft Isosensitest agar seeded with gram positive bacteria, after incubation of plates at room temperature for 3 h and then 24 h at 37°C. The anti bacterial activity of different compounds are visible as a clear zone on the red background of gram positive bacteria cells which have grown and reduced the tetrazolium chloride metabolic indicator. AMMD, *B. ambifaria* Bcc0207; BCF, *B. ambifaria* Bcc0250; KW, *B. ambifaria* Bcc1256; MEX-5, *B. ambifaria* Bcc1599; 233, *B. lata* Bcc0233; 1409, *B. vietnamiensis* Bcc1409; Pyr, pure pyrrolnitrin; Gla, pure gladiolin. The overlaid bacteria were: A, *E. faecalis*; B, *S. aureus* Egales; C, *A. baumannii*; D, *P. fluorescens*.

Table 4.2 Spectrum of inhibition of the *Burkholderia* crude resin extracts.

		Rf values of *Burkholderia* active metabolites					Controls		
Tested microorganisms		AMMD	BCF	KW	Mex-5	1409	233	Pyr	Gla
Ascomycete	*C. albicans*	(6)/ 0.0 0.09 0.44 0.65 0.85 0.98	(3) 0.03 0.61 0.98	(5) 0.09 0.52 0.65 0.84 0.98	(6)/ 0.00 0.35 0.53 0.62 0.84 0.98	(3) 0.00 0.05f 0.66 0.99	(5) 0.01 0.35 0.46 0.75 0.98	0.99	0.36
	C. parapsilosis	(6) 0.00 0.05 0.43 0.65 0.8 0.99 0.98	(2) 0.00 0.52f 0.98	(5) 0.10 0.45 0.70f 0.87 0.95 1.00	(7)/ 0.00 0.10 0.49 0.64 0.86 0.94 1.00	(4) 0.00 0.04 0.50f 0.72 0.98	(5) 0.10 0.33 0.46 0.76 0.96	1.00	0.40
Basidiomycete	*B. adusta*	(3)/ 0.02 0.70 0.9	(2) 0.02 0.98	(2) 0.02 0.98	(3)/ 0.02 0.47 0.98	N	N	0.98	N
	T. versicolor	(2) 0.02 0.98	(2) 0.02 0.98	(2) 0.02 0.98	(2) 0.02 0.98	N	N	0.98	N
	S. gausapatum	(2) 0.02 0.98	(2) 0.02 0.98	(2) 0.02 0.98	(2) 0.02 0.98	N	N	0.98	N
Gram negative bacteria	*P. fluorescens*	(6)/ 0.00 0.05 0.10 0.48 0.72 0.98	(2) 0.00 0.46f	(4)/ 0.07 0.42 0.60f 0.70f 0.86 0.99	(5) 0.00 0.41 0.69 0.85 0.98	(2) 0.00 0.07f 0.47f 0.70 0.83f	0.08f	0.98	0.38
	B. cenocepacia	0.50	0.02	-	0.02	-	-	-	0.39
	E. coli	0.4	0.0 0.5 f	-	0.0	0.0	-	1.00	0.4
	S. maltophilia	0.5	-	-	-	-	-	-	0.4
	A. baumannii	0.5	-	-	-	-	-	-	0.4
Gram positive bacteria	*E. faecalis*	(3) 0.00 0.07 0.48	(2) 0.00 0.42	(4) 0.40 0.71 0.82 0.98	(5)/ 0.0 0.45 0.74 0.82 0.97	-	(2) 0.00 0.5	0.98	0.35
	S. aureus Eagles	(2) 0.0 0.5 0.44 0.66	0.0 0.45 f	(4) 0.43 0.69 0.82 1.00	(5) 0.0 0.45 0.82 0.95 1.00	0.08	(7)0.02 0.08 0.16 0.53 0.66 0.81 0.99	1.00	0.37
	S. aureus MRSA15	(5) 0.0 0.10 0.49 0.73 0.99	(3) 0.0 0.45 1.00	(5) 0.10 0.48 0.67 0.84 0.92	(6) 0.0 0.14 0.53 0.73 0.82 0.99	(3) 0.02 0.08 0.99	(7)0.02 0.08 0.42 0.53 0.66 0.81 0.99	1.00	0.44
	S. aureus MRSAMu50.0	(4) 0.0 0.43 0.68 1.00	(2) 0.0 0.41	(4) 0.49 0.64 0.83 0.98	(6) 0.0 0.45 0.60 0.81 0.90 0.98	(2) 0.02 0.99	(7)0.02 0.08 0.16 0.53 0.66 0.81 0.99	0.99	0.35
	S. aureus NCTC12981	 0.44	0.0	(3)/ 0.71 0.83 1.00	(3)/ 0.00 0.81 1.00	-	-	0.98	0.37

Results are shown for *B. ambifaria* AMMD; BCF; KW and Mex-5; *B. vietnamiensis* 1409; *B. lata* Bcc0233; Pyr, pyrrolnitrin; Gla, Gladiolin; N, not tested; f, faint activity; R*f*, retention factor; numbers between bracts ( ) are the number of active metabolites were detected.

In addition to screening the anti-bacterial nature of the Bcc isolates, a novel pure antibiotic, designated gladiolin, was also investigated for the first time using TLC-bioautography (Figure 4.6, Gla). Gladiolin is a novel macrolide antibiotic produced by Burkholderia gladioli Bcc 0238 and is characterization has just begun (Mahenthiralingam et al., unpublished data). Gladiolin showed broad spectrum anti-bacterial activity against both Gram-positive and Gram-negative bacteria (Figure 4.5, Table 4.2).

4.3.4 Purification of *Burkholderia* active metabolites

The *Burkholderia* crude resin extracts of secreted metabolites contained large amounts of inactive materials, in addition the active antimicrobial compounds which were often present at low concentrations. To obtain large quantities of *Burkholderia* active metabolites, three purification strategies were evaluated: liquid-liquid extractions, column chromatography and silica gel preparative TLC. After multiple attempts at purification using these different methodologies, preparative TLC was judged as the most simple and productive method to purify bioactive metabolites within *Burkholderia* crude resin extracts. A preparative TLC purification scheme was developed and specifically applied to characterize the anti-candidal components of the Amberlite XAD-16 *Burkholderia* extracts (crude resin extracts). Semi-purified active *Burkholderia* fractions were subsequently sent to Warwick University for mass measurement and putative chemical identification.

4.3.4.1 Preparative TLC plates

Silica gel preparative TLC plates with two different thicknesses, 500 and 1500 µm, were used to fractionate *Burkholderia* metabolites. The thickness of the silica gel of preparative TLC had a clear affect on the fractionation of crude extracts (Figure 4.7). The 1500 µm plates showed better separation than the 500 µm plates. For example, the crude resin

extracts of *B. ambifaria* AMMD, which contained 2 major active metabolite spots on both plates, showed much greater separation on the 1500 µm plate (Figure 4.7). The crude resin extracts of *B. ambifaria* KW and Mex-5 gave, respectively, 5 and 6 active metabolites, well separated anti-candidal metabolites on plates of 1500 µm thickness; in comparison, these strains gave, respectively, just 3 and 4 active anti-candidal metabolites on plates of 500 µm which showed a reduced ability to separate active components within the same crude extracts (Figure 4.7). Additionally, the 1500 µm preparative TLC plates also possessed about four times the loading capacity, and larger quantities of the crude resin extracts could be inoculated to provide better yields of the active fractions.

Once the anti-candidal components of the preparative TLC fractions had been identified using the TLC-bioautography assays, replicate separations were set up where a single strain extract was fractionated on a single TLC plate. Using the R*f* values observed in the bioassays (Figure 4.7), area of silica gel sorbent containing the active metabolites was scraped and collected. Absolute methanol was then used to re-extract semi-pure *Burkholderia* metabolites bound to silica gel; after filtration to remove the gel, the extract was concentrated and stored at -20ºC for further analysis.

From the analytical and preparative TLC it was clear that *B. ambifaria* BCF and Mex-5 produced metabolites associated with highly active zones that did not migrate under the TLC conditions applied. These spots were dark brown in colour and their anti-candidal activity was considerable (Figure 4.7). Fractionation of these basal fractions was therefore

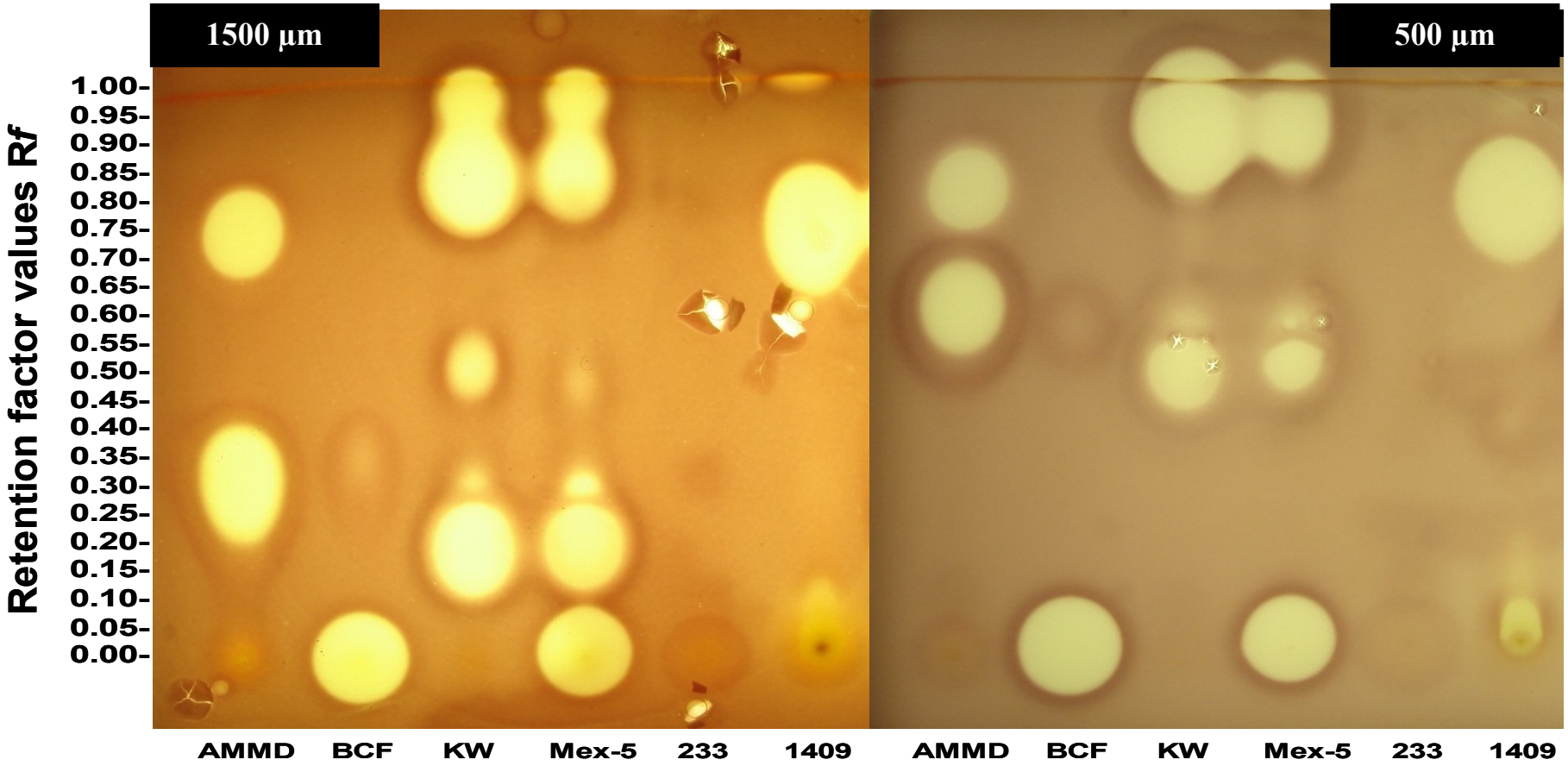


Figure 4.7 Fractionation and purification of anti-candidal agents of *Burkholderia* using preparative silica gel TLC.

Crude resin extracts of 6 *Burkholderia*, were spotted onto preparative TLC plates with different thickness 500 µm and 1500 µm and developed with ethyl acetate: methanol: water (20:1:0.5). The developed plates were then overlaid with soft Isosensitest agar seeded with *C. albicans*. After incubation of plates at room temperature for 3 h and then 24 h at 37°C. The anti-candidal activity of different compounds was visible as a clear zone on the red background of Candida cells which have grown and reduced the tetrazolium chloride metabolic indicator. AMMD, *B. ambifaria* Bcc0207; BCF, *B. ambifaria* Bcc0250; KW, *B. ambifaria* Bcc1256; MEX-5, *B. ambifaria* Bcc1599; 233, *B. lata* Bcc0233 and 1409, *B. vietnamiensis* Bcc1409.

attempted using different solvents with different polarities to see if they could be separated further. A mixture of ethylacetate:methanol:water (4:1:0.25) was able to fractionate these non-migrating spots into roughly 3 different fractions with anti-candidal activity (R*f*s 0.05, 0.30 and 0.65; Figure 4.8).

The separations were not as clear as seen under previous conditions (Figure 4.7), with active metabolites running as more of a smear, however, a non-migrating zone and two inhibitions zones above this could be seen. Moreover, these results also confirmed that the single inhibition zones seen in the TLC fractionation of *Burkholderia* extracts may sometimes be due to the presence of multiple active metabolites.

Semi-pure fractions of *Burkholderia* crude resin extracts were evaluated against *C. albicans* using TLC-Bioautography assays, to detect the activity and purity of each fraction. The fractionation progression using 1500 µm preparative TLC plates was able to separate reasonable amounts of semi-pure compounds for further investigations. The crude extract of *B. ambifaria* AMMD provided 3 semi pure active fractions which separated at different R*f*s 0.66, 0.85 and 0.98 (Figure 4.9). The *B. ambifaria* BCF extract provided 4 semi pure active fractions that were located at R*f*s 0.00, 0.20, 0.60 and 0.98 (Appendix B: Figure B3; N1, T1). The *B. ambifaria* KW extract provided 5 semi pure active fractions (Appendix B: Figure B3; N2, T2) separating R*f*s 0.00, 0.50, 0.60, 0.84 and 0.98. *B. ambifaria* Mex-5 extract provided 6 semi pure active fractions which were located at R*f*s 0.00, 0.25, 0.50, 0.74, 0.84 and 0.98 (Appendix B: Figure B4). The activity of these fractions was retested against *C. albicans* and the activity was confirmed.

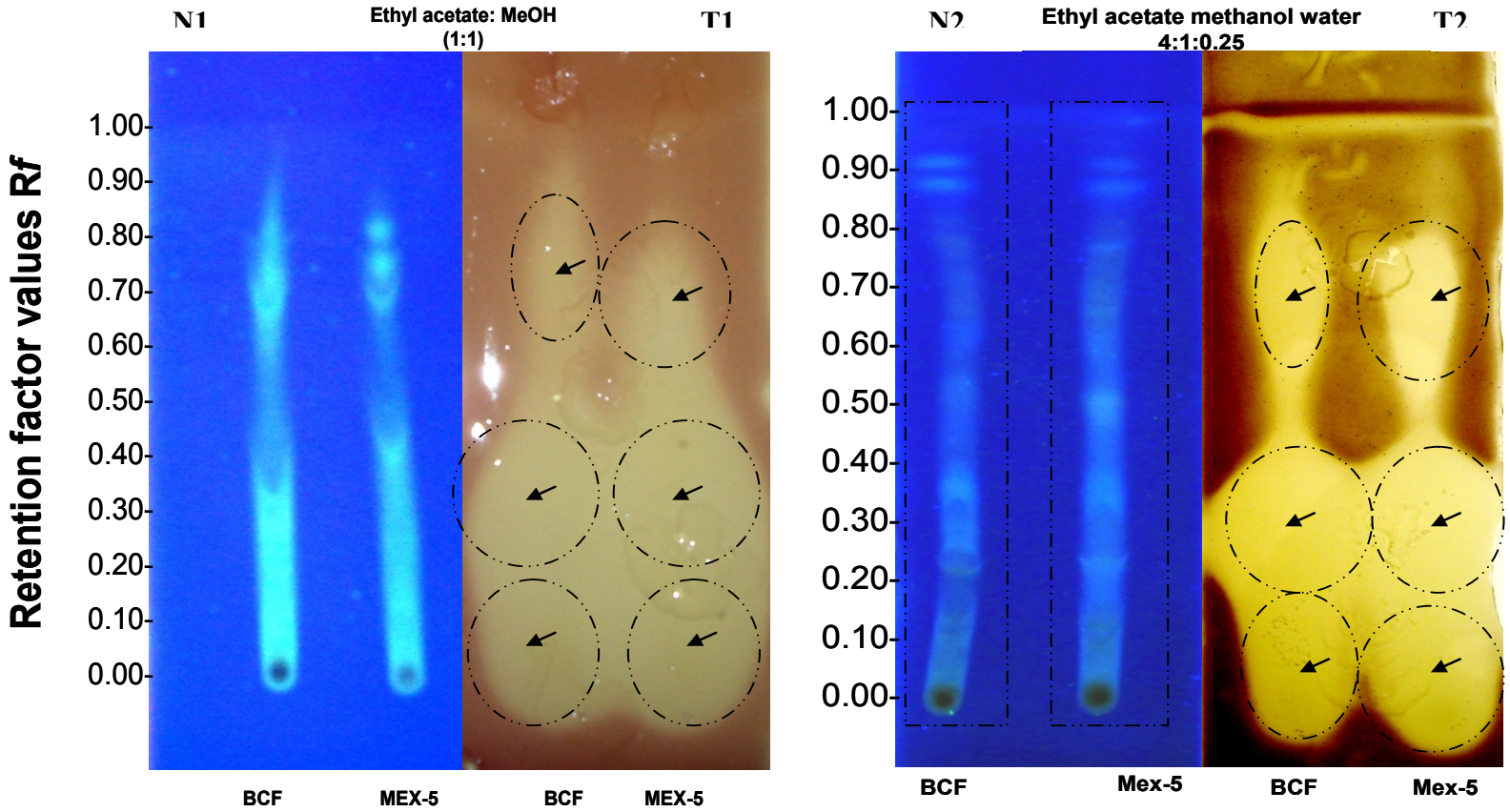


Figure 4.8 Fractionation and detection of anti-candidal activity of the previously non-migrating active metabolites (R*f* 0.01) of *B. ambifaria* BCF and Mex-5.

Non-migrating fractions (R*f* 0.01) of *B. ambifaria* BCF and Mex-5, were spotted onto TLC plates in duplicate. One set of TLC plates was developed with ethyl acetate: methanol (1:1) (panel N1) and the other set was developed with ethyl acetate:methanol:water (20:1:0.5) (panel N2). The developed TLC plates were then overlaid with soft Isosensitest agar seeded with *C. albicans*. After incubation of plates at room temperature for 3 h and then 24 h at 37°C, the anti-candidal activity of different compounds was visible as a clear zone on the red background of *Candida* cells which have grown and reduced the tetrazolium chloride metabolic indicator (panel T1 and T2). Arrows indicated the location of active semi-pure metabolites.

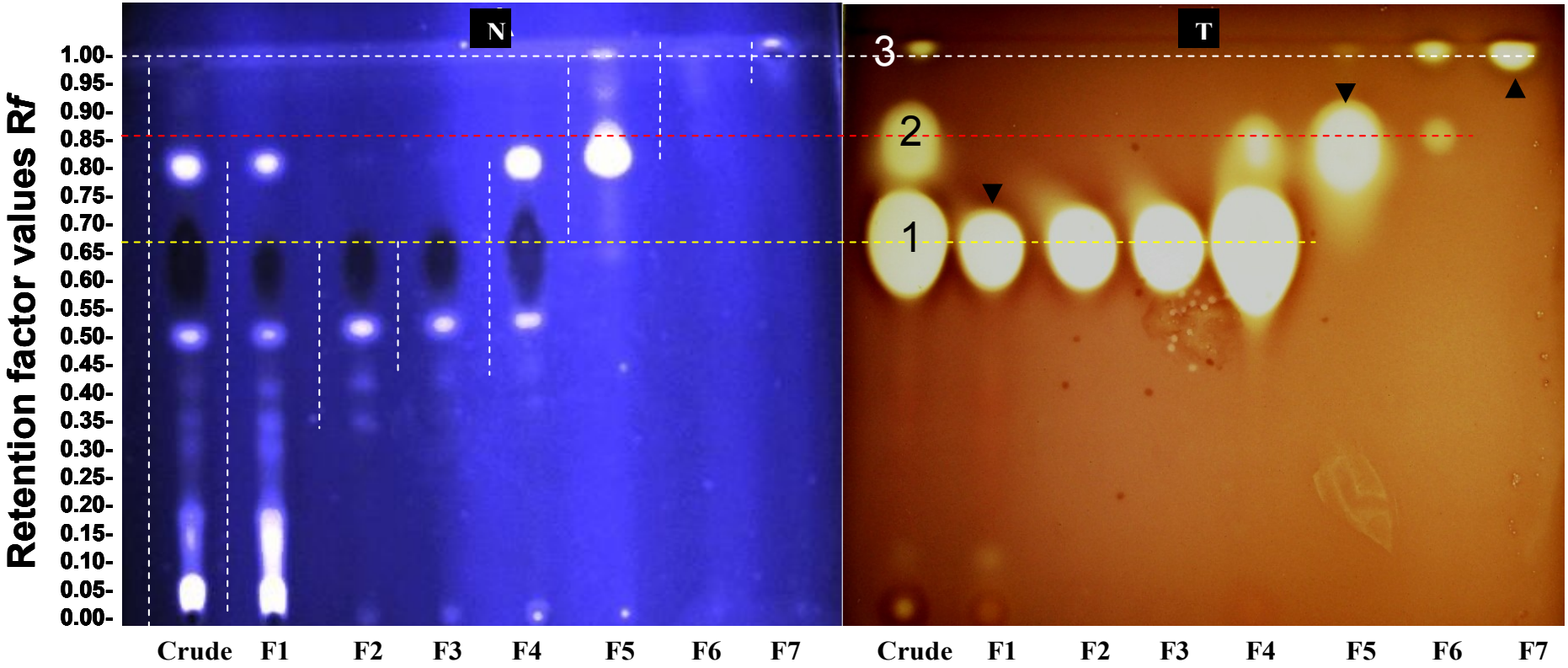


Figure 4.9 Profile of *B. ambifaria* AMMD metabolites fractionated using silica gel preparative TLC.

Semi-pure fractions (Fraction 1 to fraction 7) fractionated from crude resin extract and cured resin extract of *B. ambifaria* AMMD were spotted onto TLC plates in duplicate. One set of TLC plates was developed with ethyl acetate: methanol: water (20:1:0.5) (panel N). The other set was overlaid with soft Isosensitest agar seeded with *C. albicans*. After incubation of plates at room temperature for 3 h and then 24 h at 37°C, the anti-candidal activity of different compounds was visible as a clear zone on the red background of *Candida* cells which had grown and reduced the tetrazolium chloride metabolic indicator (panel T). Arrows indicate the location of active semi-pure metabolites.

4.3.4.2 Liquid-liquid extraction

The liquid-liquid method of fractionation was also evaluated for use on *B. ambifaria* AMMD crude extracts using different solvents with different polarities in an attempt to purify component antimicrobials (section 2.11.1). Anti-candidal activity was found in just one fraction of *B. ambifaria* AMMD extract, a yellow fraction extracted using the non-polar solvent di ethyl ether. TLC-bioautography indicated that the activity of the ether fraction was due to just one yellow compound which lay at R*f* 0.65 (data not shown). In contrast, the results of the TLC-bioautography assay of AMMD crude extract revealed that the anti-candidal activity located to at least 6 inhibition zones seen at different R*f*s (Table 4.2).

4.3.4.3 Column chromatography

The *B. ambifaria* AMMD crude resin extract was also fractionated using silica gel column chromatography into 4 fractions with 3 active anti-candidal metabolites using ethyl acetate :methanol:water 20:1:0.5 (Figure 4.10). The first active metabolite collected from the beginning of the eluting process within the first 10 ml of solvent and deposited at R*f* 0.98, was colourless under day light and black under UV at 366 nm (Figure 4.10, G1). The second active metabolite collected after 15 ml until 25 ml from the beginning of the fractionation process and deposited at R*f* 0.73 ± 0.01, was colourless under day light and light blue under UV at 366 nm (Figure 4.10, G2). The third active metabolite detected at 30 ml of eluted solvent and collected as a pure compound after 100 ml until 475 ml from the beginning of the fractionation process and deposited at R*f* 0.54 ± 0.02, was yellow under day light and black under UV at 366 nm (Figure 4.10, G3).

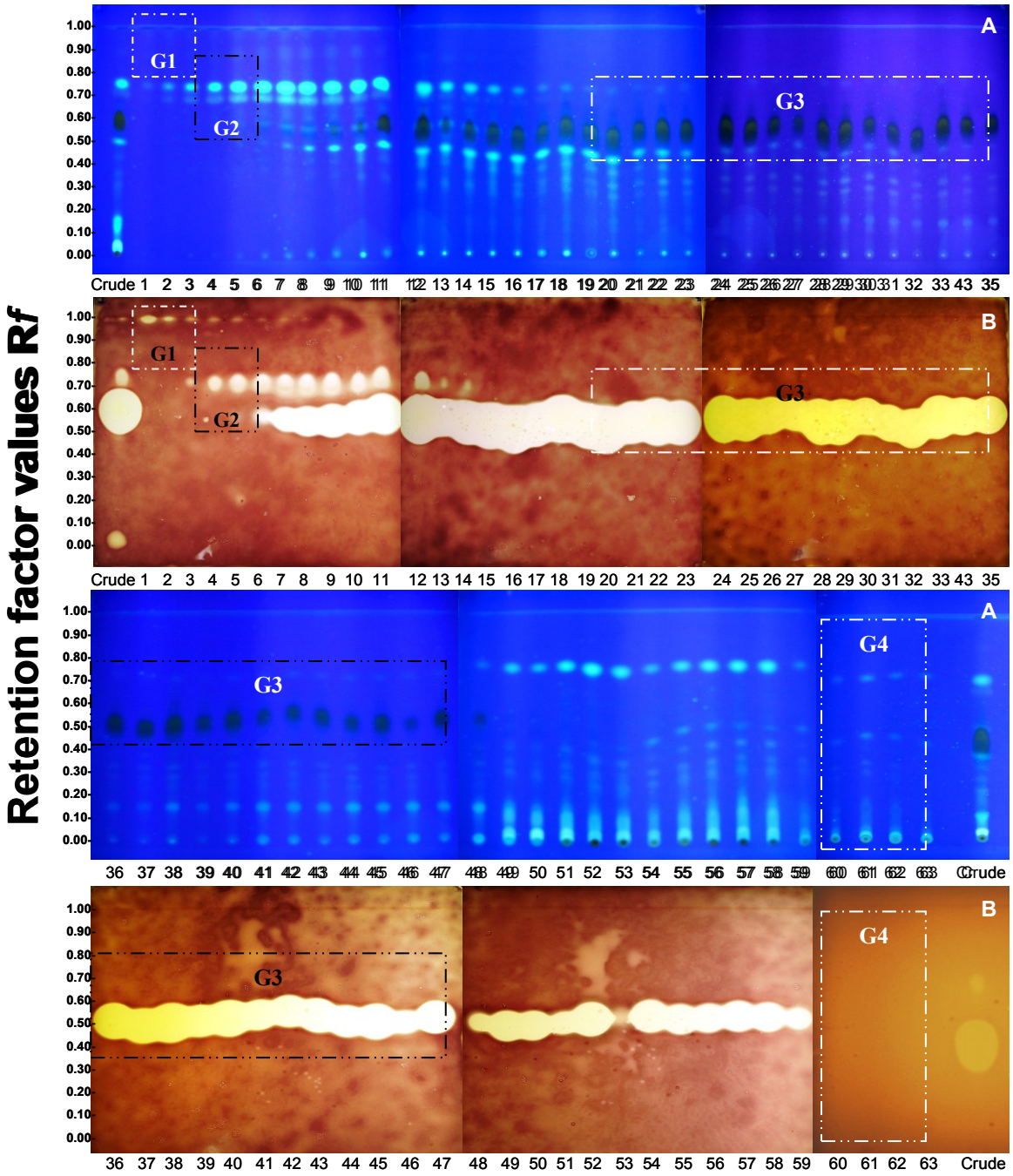


Figure 4.10 Profile of *B. ambifaria* AMMD metabolites fractionated using silica gel column chromatography.

B. ambifaria AMMD crude resin extract was fractionated by ethyl acetate, methanol and water 20:1:0.5 using silica gel chromatography column. The fractions were collected and then analysed using TLC-bioautography as shown. Semi-pure fractions (Fraction 1 to fraction 63) fractionated from cured resin extract of *B. ambifaria* AMMD were spotted onto TLC plates in duplicate. One set of TLC plates was developed with ethyl acetate: methanol: water (20:1:0.5) (panel A). The other set was overlaid with soft Isosensitest agar seeded with *C. albicans*. After incubation of plates at room temperature for 3 h and then 24 h at 37°C, the anti-candidal activity of different compounds was visible as a clear zone on the red background of *Candida* cells which have grown and reduced the tetrazolium chloride metabolic indicator (panel B). G1, first active semi-pure metabolites (R*f* 0.99); G2, second active semi-pure metabolites (R*f* 0.70); G3, third active semi-pure metabolites (R*f* 0.52); G4, forth in active semi-pure metabolites; crude, crude risen extract.

4.3.5 Identification of anti-candidal compounds produced by *B. ambifaria* isolates

Semi-pure fractions of *B. ambifaria* isolates AMMD, BCF, KW and Mex-5 (section 2.11.2) were sent to Warwick University for characterisation and identification.

4.3.5.1 Anti-candidal compounds produced by *B.* ambifaria AMMD

B. ambifaria AMMD produced 6 active fractions (Figure 4.4). The basal fraction (R*f* 0.01) provided 3 ions with m/z of 758.3905, 776.4010, 790.3182 with molecular formulas of C45 H52 N5 O6, C45 H52 N5 O7 and C25 H48 N11 O18, respectively (Appendix B; Figure B5). It was proposed that these ions correspond to polyethylene glycol (PEG), unknown peptides and plasticizer respectively. Fraction 2 which had R*f* 0.65 provided (Figure 4.11, a) an ion with m/z = 724.2270 with molecular formulas (C33 H45 Cl 2 N Na O11). It was deduced that these ions correspond to enacyloxin II a.

Fraction 3 which had R*f* 0.86 provided 3 ions with m/z 256.1705, 256.1693 and 256.1693 with molecular formulas C17 H22 N O, C19 H26 N O and C21 H30 N O3, respectively (Figure 4.11, b). It was deduced that these ions correspond to a group of quinolinones: 4-hydroxyl-3-methyl-2-alkyl-4-quinolinone (HMAQ-256), 4-hydroxyl-3-ethyl-4-quinolinone (HAQ-284) and one other derivative of 4-hydroxyl-3-methyl-2-alkyl-4-quinolinone (HMAQ-344). Fraction 4, which deposited at R*f* 0.98, provided ions with m/z = 301.1409 and 413.2664 and molecular formulas of C16 H22 Na O4 and C24 H38 Na O4, respectively (Figure 4.11). It was deduced that these ions correspond to different plasticizers.

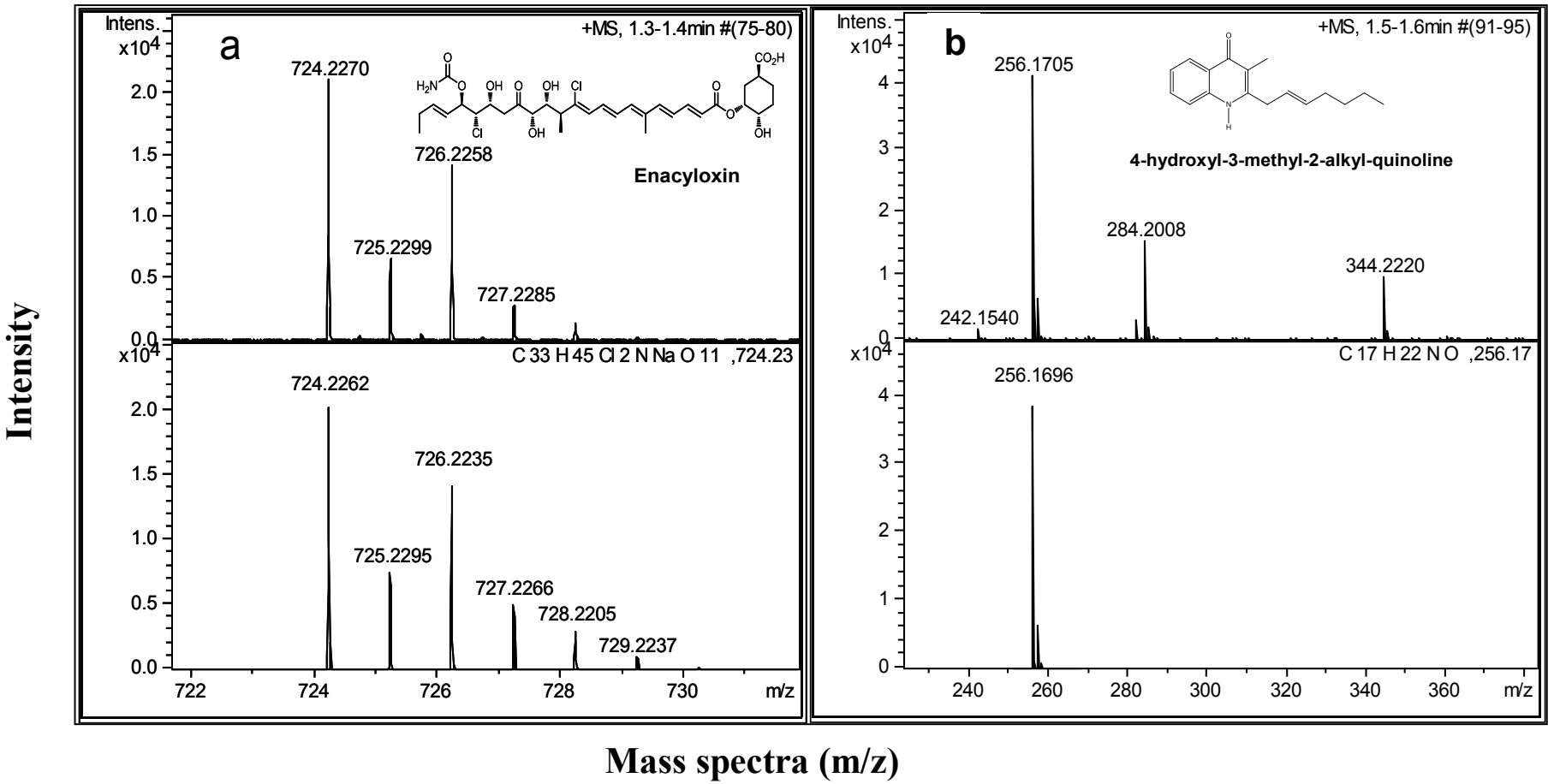


Figure 4.11 Mass spectra of *B. ambifaria* AMMD fraction 3 (R*f* 0.65) and fraction 4 (R*f* 0.48).

Data indicated that these fractions were dominated by (a) enacyloxin IIa (724 m/z) and other putative enacyloxin isomer (b) 4-hydroxyl-3-methyl-2-alkyl-quinoline (HMAQ-256), 4-hydroxyl-3-ethyl-quinoline (HAQ-284); and derivative of 4-hydroxyl-3-methyl-2-alkyl-quinoline (HMAQ-344).The chemical structure shown on lift panel (a) is enacyloxin IIa, and the chemical structure shown on right panel (b) is HMAQ respectively.

4.3.5.2 Anti-candidal compounds produced by *B. ambifaria* BCF

B. ambifaria BCF provided 3 active fractions with anti-candidal activity. The BCF basal fraction was deposited at R*f* 0.01 and provided 4 ions with m/z 454.1164, 438.1211, 383.0755 and 494.1466, with molecular formulas C19 H28 Cl 2 O8, C19 H28 Cl 2 O7, C15 H17 Cl 2 N6 O2 and C20 H30 Cl 2 N3 O7, respectively (Figure 4.12). It was proposed that these ions correspond to different bactobolins: A, B, D and a new bactobolin containing Cl_2 (Figure 4.12, d). Fraction 2 was deposited at R*f* 0.61 and provided an ion with m/z = 724.2270 and with a molecular formula of C33 H45 Cl 2 N Na O11. It was deduced that these ions corresponded to enacyloxin II a. Fraction 3 was deposited at R*f* 0.98 and provided ions with m/z = 301.1409 and 413.2664, and molecular formulas C16 H22 Na O4 and C24 H38 Na O4. It was deduced for these ions that they correspond to different plasticizers as had been seen with the AMMD fraction 7 analysis.

4.3.5.3 Anti-candidal compounds produced by *B. ambifaria* KW and Mex-5

The crude extract of *B. ambifaria* KW and Mex-5 provided 5 and 6 active anti-candidal fractions respectively. The mass spectra of these fractions indicated the presence of at least 10 potentially novel anti-candidal metabolites (Appendix B; Figures B7 and B8). The basal fraction of *B. ambifaria* Mex-5 was deposited at R*f* 0.01 and provided many ions with m/z 383.0770, 367.0824 and 395.0780 with molecular formulas C14 H21 Cl2 N2 O6, C14 H21 Cl2 N2 O5 and C15 H21 Cl2 N2 O6, respectively (Figure 4.13). As had been observed with strain BCF, these were putatively identified as derivatives of bactobolin.

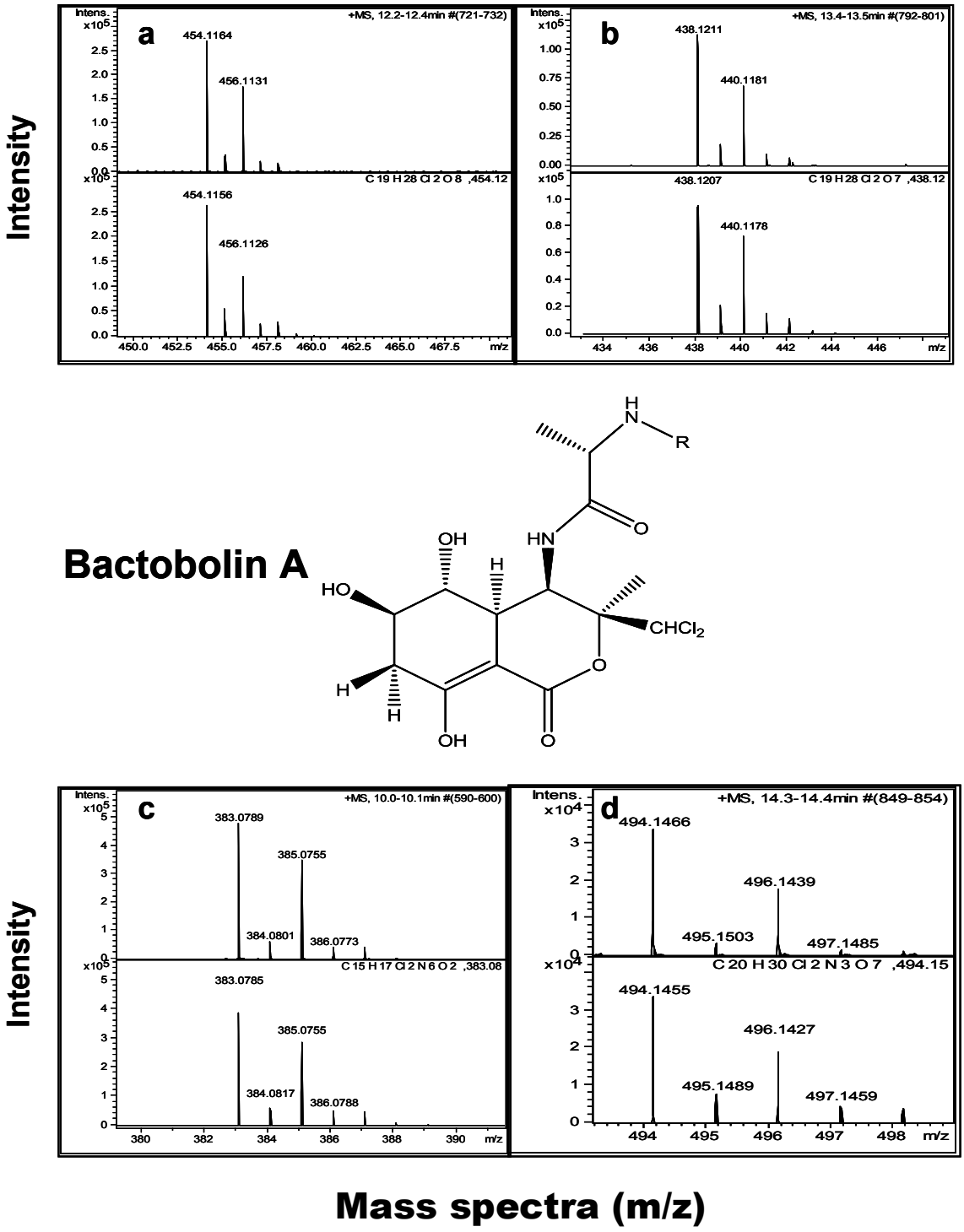


Figure 4.12 Mass spectra of *B. ambifaria* BCF fraction 1 (R*f* 0.01).

Data indicated that this fraction is dominated by different of Bactobolins. (a) Bactobolin A; (b) Bactobolin B; (c) Bactobolin D; and (d) new Bactobolin containing Cl2. The chemical structure shown on the middle of the panel is bactobolin A.

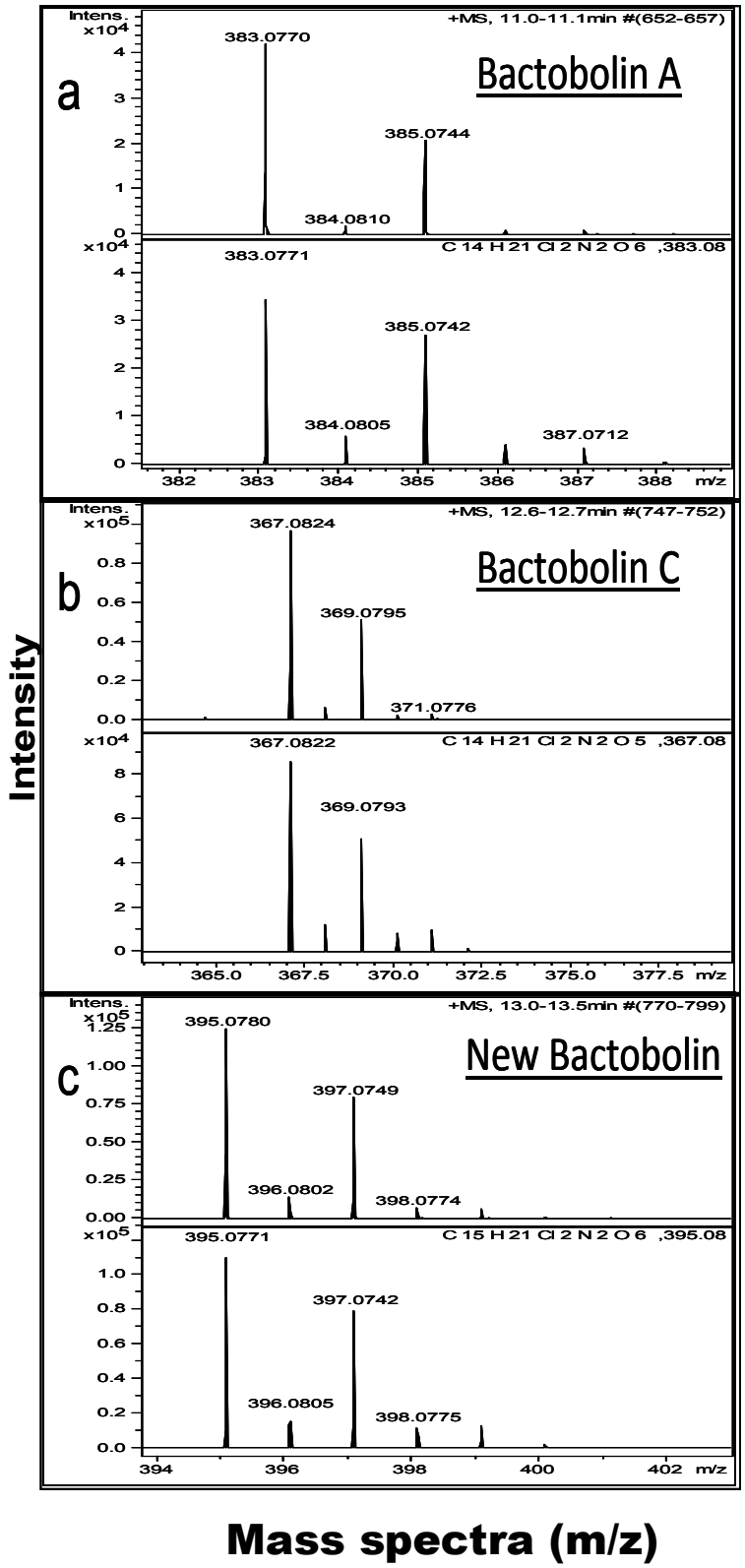


Figure 4.13 Mass spectra of *B. ambifaria* Mex-5 fraction 1 (Rf 0.01).

Results indicated that, fraction 1 was dominated by many of Bactobolins. (a), Bactobolin A (383.077 m/z); (b), Bactobolin c (367.08 m/z) and (c), new Bactobolin (395.078 and 397.07 m/z).

It was proposed that these ions correspond to bactobolin A, C and a new bactobolin containing Cl_2 (Figure 4.13, c). The Mex-5 bactobolins were different in mass to those produced by strain BCF, except for the BCF bactobolin D and Mex-5 bactobolin A which shared very similar ion mass. The results also indicated that bactobolin C was the major bactobolin fraction detected in the Mex-5 metabolites. Mass spectra data indicated that *B. ambifaria* KW and Mex-5 produced many compounds but database search indicated these belong to new chemicals. Overall, the mass spectra data indicated that both *B. ambifaria* KW and Mex-5 produced a wide range of known bioactive metabolites such as the bactobolins as well as novel compounds with mass that did not match to those within the database of antimicrobials examined at Warwick University.

4.3.6 Detection of pyrrolnitrin in *B. ambifaria* and comparison of extraction procedures

Several previous studies on *Burkholderia* sp have shown that pyrrolnitrin was one of the compounds they produce that contribute to their antifungal activity (Schimdt, *et al.*, 2009). Therefore, in the current study pure pyrrolnitrin (5 µg) was included as a known control for comparison with *Burkholderia* anti-candidal metabolites and incorporated into the TLC and other purification schemes used to isolate novel metabolites.

TLC fractionation of pyrrolnitrin using the ethyl acetate: methanol: water (20:1:0.5) solvent system results in the compound migrating with the solvent front at R*f* values of greater than 0.95. Therefore, other solvent systems were evaluated based on those that had been previously applied to pyrrolnitrin (Burkhead, *et al.*, 1994). Silica gel TLC-bioautography assay using a chloroform-ethyl acetate-formic acid 5:4:1 v/v solvent system

was successful for fractionation of pyrrolnitrin. The pure compound located as a single dark spot at R*f* 0.77 (Figure 4.14, N1 pyr), produced a large clear inhibition zone (12mm) against *C. albicans* (Figure 4.14, T1 pyr). In addition using fluorescence TLC plates and chloroform:acetone (9:1) as the solvent system, pure pyrrolnitrin gave a single dark spot at R*f* 0.685 (Figure 4.14, N2 pyr) and a large (18 mm) inhibition zone (Figure 4.14, T2 pyr). Both systems were, therefore, applied to screen the *Burkholderia* extracts for the presence of pyrrolnitrin-like metabolites.

Several fractions from the metabolite isolation and purification work were suspected to contain pyrrolnitrin as one of the active antifungal metabolites. The last fraction of preparative TLC plates of *B. ambifaria* AMMD crude extract, which was deposited at R*f* 0.98 (Table 4.2), co-located adjacent to pure pyrrolnitrin (Figure 4.14). The first fraction of column chromatography, the ethyl acetate fraction (Figure 4.10), also ran at the same R*f* as pure pyrrolnitrin (Figure 4.14). Finally, an acetone extract from the liquid-liquid extractions also showed the same results as pure pyrrolnitrin (Figure 4.14). All these AMMD fractions also produced the anti-candidal activity characteristic of pyrrolnitrin under both conditions of TLC fractionation (Figure 4.14: T1 and T2). The same separation scheme was also applied to the last preparative TLC fraction (R*f* 0.98) of *B. ambifaria* Mex-5, and the isolated active compound showed the same R*f* and anti-candidal activity as pure pyrrolnitrin under both conditions of the preparative TLC plate. Overall, these results demonstrated that both *B. ambifaria* AMMD and Mex-5 produced an antifungal compound that was most likely pyrrolnitrin, a well characterized and highly bioactive *Burkholderia* metabolite.

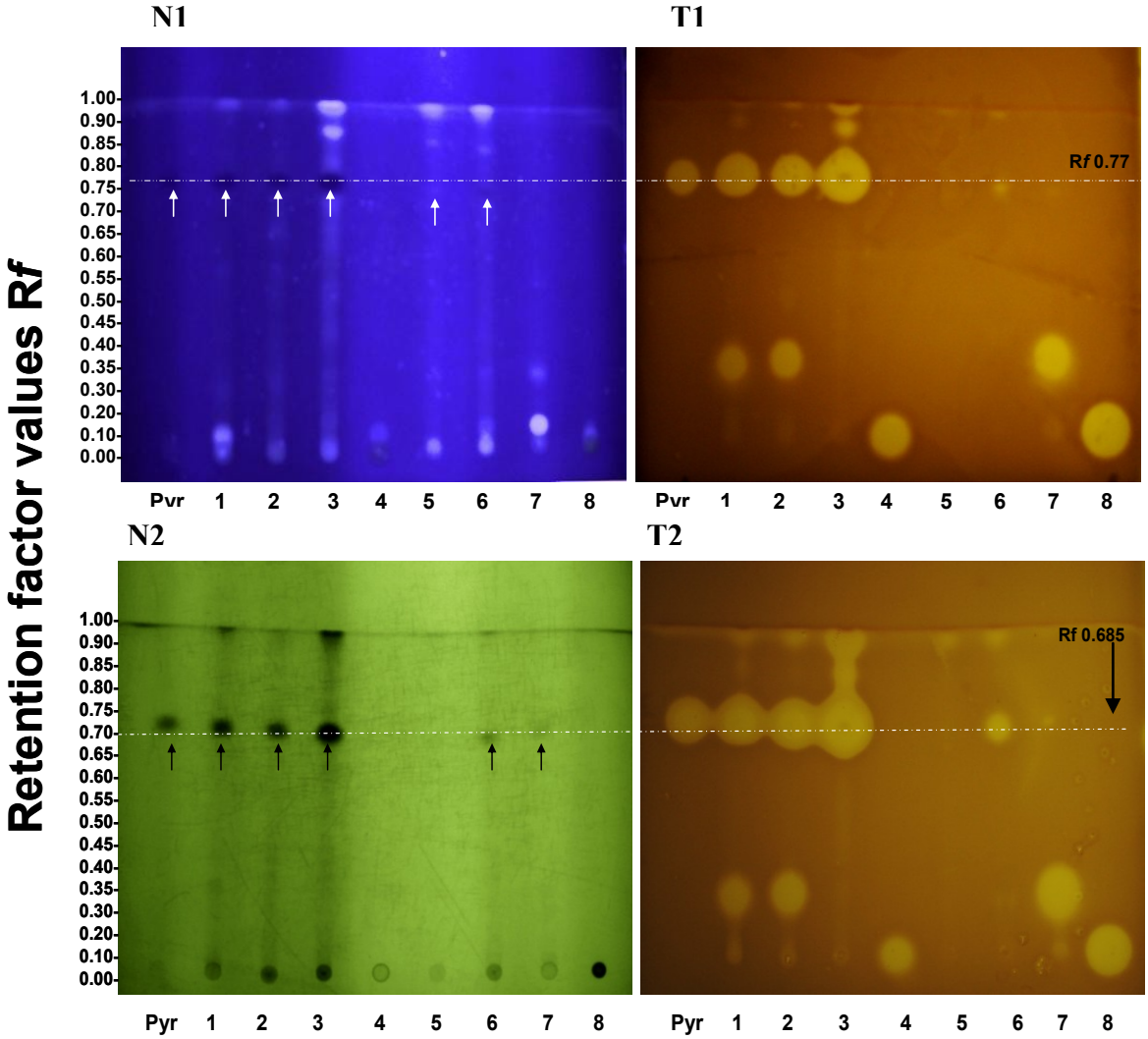


Figure 4.14 Characterisation of pure pyrrolnitrin and semi-purified antifungal extracts of *B. ambifaria* using two different systems of TLC and solvents.

The first system was normal silica gel (0.25 µm) and chloroform-ethyl acetate-formic acid 5:4:1 v/v as solvent system (N1) and the second system was fluorescens silica gel (0.25 µm) and chloroform: acetone 9:1 v/v as solvent system (N2). One set of TLC plates was visualised using UV at 366 nm (N1 & N2) and the other set was overlaid with *C. albicans* (T1 & T2). Pyr, pure pyrrolnitrin (Sigma); 1, *B. ambifaria* AMMD semi-pure fraction of preparative TLC (R*f* 0.98); 2, *B. ambifaria* AMMD first fraction of column chromatography called ethyl acetate fraction; 3, acetone extract of liquid extraction; 4, *B. ambifaria* BCF semi-pure fraction of preparative TLC (R*f* 0.98); 5, *B. ambifaria* KW semi-pure fraction of preparative TLC (R*f* 0.98); 6, *B. ambifaria* Mex-5 semi-pure fraction of preparative TLC (R*f* 0.98); 7, *B. ambifaria* AMMD second fraction of column chromatography called the ethyl acetate: methanol (1:1) fraction; 8, *B. ambifaria* AMMD third fraction of column chromatography called methanol fraction. Arrows indicate the location of pure pyrrolnitrin and similar metabolites (R*f*) of other extracts.

4.3.7 Determination of antimicrobial activity of crude and semi purified extracts of *Burkholderia* sp

The antimicrobial activity of *Burkholderia* crude and semi purified extracts were further evaluated using broth dilution assay against a range of microorganisms. A minimal inhibitory concentration (MIC) was calculated for each *Burkholderia* extract against each respective susceptibility testing organism (Table 4.3). The MICs ranged from 15.6 to 5000 µg/ml and demonstrated a wide spectrum of activities against *C. albicans*, Gram-positive and Gram-negative bacteria; extracts which were active on all three test groups were considered as broad spectrum. Both the crude resin extract and acetone extract of *B. ambifaria* AMMD were active against *C. albicans*, Gram-positive and Gram-negative bacteria. Crude resin extracts of *B. ambifaria* AMMD and Mex-5 were more active against Gram-negative bacteria than the comparable acetone extracts. In particular, the crude resin extracts of *B. ambifaria* AMMD were 20 times more potent than acetone extracts against *B. multivorans* and *E. coli* (Table 4.3 and Appendix B; Figure B9).

The presumptive enacyloxin IIa of *B. ambifaria* AMMD (Fraction 2) was very active against all tested microorganisms: *B. multivorans*, *E. coli*, *S. aureus*, *C. albicans* and *P. aeruginosa* and MIC values were 15.6, 15.6, 62.5, 125 and 250 µg ml^{-1} respectively. The active concentrations of AMMD crude resin extract were 187.5, 375, 1500, 1500 and 5000 µg ml^{-1} respectively for the same test organisms. The *B. ambifaria* BCF crude resin extract was also very active against the gram-negative *B. multivorans* and *E. coli,* with MIC values were 46.8 µg ml^{-1}. BCF crude resin extract was more active than the equivalent extracts from *B. ambifaria* AMMD for all tested organisms except *P. aeruginosa* (Table 4.3).

Table 4.3 Minimum inhibitory concentrations of *Burkholderia* antimicrobial extracts

Extracts [b] (µg ml^{-1}) / Microbes [a]	*B. ambifaria* AMMD extracts			Mex_5		BCF	KW	*B. gladioli*
	Crude resin extracts	Acetone extract	Semi-pure fraction (F6*)	Crude resin extracts	Acetone extract	Crude resin extracts	Crude resin extracts	Crude resin extracts
C. albicans	1500	> 5000	125	3000	>5000	3000	1500	46.8
S. aureus	1500	1500	62.5	1500	>5000	750	3000	750
B. multivorans	375	1500	15.6	187.5	>5000	46.8	1500	187.5
E. coli	187.5	>5000	15.6	187.5	>5000	46.8	375	187.5
P. aeruginosa	>5000	>5000	250	< 5000	>5000	>5000	>5000	>5000

MIC values are giving as µg/ml for crude and semi-pure extracts of *Burkholderia ambifaria* AMMD, BCF, KW and Mex_5, and *B. gladioli* BCC0238. a; The susceptibility testing organisms were *C. albicans* (Sc5314), *Staphylococcus aureus* (NCTC12981), *B. multivorans* (ATCC17210), *Escherichia coli* (NCTC12241) and *Pseudomonas aeruginosa* (NCTC12903). b; The semi-pure fraction F6 extracted from the AMMD extract using silica gel column chromatography had been shown to contain enacyloxin IIa. *; Semi pure fraction of *B. ambifaria* AMMD crude resin extract which was fractionated using preparative silica gel TLC.

4.4 Discussion

The TLC-Bioautography assay under the current experimental conditions showed that *Burkholderia* produced a large variety of active secondary metabolites which exhibited a broad spectrum of activity against Gram-positive and Gram-negative bacteria, yeasts and basidiomycetes. This result was unpredicted and could not be achieved using other antimicrobial assays. We have performed an Amberlite XAD 16 resin extraction procedure for the first time on *Burkholderia* antimicrobials, using methanol as the elution solvent; this method was selected as the best to use because of its simplicity and the extract purity obtained.

Several different TLC visualization methods were used in this study; UV light was the simplest to apply and allowed multiple metabolites to be located. This corroborates the findings of the previous work in this field (Kadir*, et al.*, 2008) recommending UV light as a visualization method. UV light at 366 nm was the superior visualization method for *Burkholderia* resin extracts which were separated on standard silica gel TLC plates. To obtain the best results, visualization was performed with the silica gel side of the TLC plate face down on the source of the UV light. Under these conditions the resin extracts of *Burkholderia* produced highly distinctive profiles of *Burkholderia* metabolites. Care must be taken when obtaining these photographs to avoid excessive exposure to UV radiation.

Ethyl acetate, methanol and water (EtOH:MeOH:H_2O), with both ratios 10:1:0.5 and 20:1:0.5, was one of the best solvent systems identified to resolve *Burkholderia* metabolites. Chloroform and acetone (1:1), and methanol and water (8:2), were also able to

partition the extracts under investigation, but neither performed quite as well as the Ethyl acetate, methanol and water mixture. This could be because the solvent system was a mixture of three different solvents with different polarities. Ethyl acetate, methanol and water combination had a strong ability to partition metabolites and the active anti-microbials within the crude *Burkholderia* extracts that were polar and semi-polar compounds.

Two other steps in the development of the TLC-bioautography assay which were found to be critical for successful analysis were as follows. The fractionated TLC plates had to be exposed for at least 15 min of aeration within a chemical fume cupboard before the agar overlay was added. If the residual solvents were not removed in this way they often inhibited microbial growth in the overlay. Another critical finding was that the pre-incubation of the overlaid TLC plates for at least 3 hours at room temperature was optimal to allow diffusion of *Burkholderia* metabolites from the silica gel into the seeded agar layer. This pre-incubation allowed very clear inhibition zones to be observed for antimicrobial agents present on the developed TLC plates. This finding corroborates the ideas of Rojas *et al*., (2008), who suggested that the pre-incubation of plates for up to 12 h was necessary to achieve clear results from an antimicrobial suitability test. Consistency of these simple steps in performing bioautography assay were important, especially if the assay was going to be used as a preliminary screening technique to detect antimicrobial components of novel *Burkholderia* extracts.

The TLC-bioautography assay was able to provide data which helped with the interpretation of findings from the simple overlay assay used to screen for antimicrobial

activity (see chapter 3). For example, *B. ambifaria* Bcc0193 demonstrated no anti-candidal activity in the overlay screens and hence was selected as a negative control for the metabolite extraction analysis; the TLC-bioautography assay also confirmed that this Bcc isolate did not produce highly active metabolites, with only very a faint affect on *C. albicans* seen after TLC analysis of concentrated metabolite extracts from Bcc0193 (Figure 4.5). *B. cenocepacia* Bcc0019 was able to inhibit the growth of *C. albicans* when grown on SA medium but did not produce this affect when grown on BSM-G medium. The TLC-bioautography assay was able to explain the effect of different media on anti-candidal production, locating the presence of one active metabolite produced by *B. cenocepacia* Bcc0019 when it was grown on SA medium (Figure 4.5). This could be because this compound was induced after growth on SA remains to be determined.

The TLC-bioautography, LC-MS data and comparative analysis of *B. ambifaria* resin extracts showed that *Burkholderia* are potentially a very large reservoir of known and novel antimicrobial agents, especially with those exhibiting anti-fungal activity. Many of these compounds were discovered for the first time in this group of bacteria. The *B. ambifaria* AMMD resin extract comprised five main compounds. The following were detected previously in other *Burkholderia* sp. (1) polyethylene glycol, (2) quinolines group (4-hydroxyl-3-methyl-2-alkyl-4-quinoline, 4-hydroxyl-3-ethyl-4-quinoline and other HMAQ) and (3) pyrrolnitrin. Enacyloxin II a, a known antibiotic previously found in a *Frateuria* species bacterium, was identified for the first time to be also produced by *B. ambifaria* (Mahenthiralingam, *et al*., 2011). The presence of novel unknown peptide antibiotics in strain AMMD also shows further antimicrobials may be discovered in this strain.

B. ambifaria BCF produced 4 active bands which exhibited nearly the same profile as *B. ambifaria* AMMD, except *B. ambifaria* BCF produced a highly polar basal fraction (R*f* 0.05) which was identified as a mixture of bactobolins: bactobolin A, B and D (Kondo*, et al.*, 1979, Seyedsayamdost*, et al.*, 2010) and a new bactobolin containing Cl_2. *B. ambifaria* KW and Mex-5 produced more than 6 active bands containing at least 10 detectable active compounds. Most of these potent compounds could not be fully identified and were presumed to be unknown antifungal agents. The basal fraction of *B. ambifaria* Mex-5 (R*f* 0.05), that was identified as a mixture of new bactobolins, was different compared with the BCF strain. Bactobolin C was the major compound detected in Mex-5, but bactobolin D and B were not detected, while a new bactobolin was observed.

The detection of pyrrolnitrin within the resin extracts of *Burkholderia* using LC-MS failed. This might be due to the limited amount of pyrrolnitrin produced by *Burkholderia* under these growth and extraction conditions. Also a large amount of plasticizer was identified in some extracts and this fractionated in a very similar way to pyrrolnitrin running with the solvent front; after this was discovered use of plastics was avoided in the extraction. A method using pure pyrrolnitrin as a control and previous conditions for its detection by TLC-bioautography was able to indicate that pyrrolnitrin-like metabolites were present in several *Burkholderia* strains. Ethyl acetate, methanol and water (20:1:0.5) produced the best overall fractionation of *Burkholderia* crude extracts, however, pyrrolnitrin travelled with the solvent system until the top of solvent front (R*f* around 0.97 ± 0.02) and hence it was not useful to isolate this antifungal. Therefore, different solvent systems and different TLC sorbents were used, as recommended by Burkhead, *et al*., (1994) and Schmidt, *et al*., (2009), to detect and improve the isolation of pyrrolnitrin by TLC. The findings of the

current study support the previous studies that reported that *Burkholderia* were considered well known for their ability to produce pyrrolnitrin (Arima*, et al.*, 1964, Cartwright*, et al.*, 1995). Pyrrolnitrin showed a strong effect on *C. albicans* and basidiomycetes; it was also active against Gram-positive bacteria, but did not show inhibitory effect on many of the Gram-negative bacteria. The findings were similar to the results of previous studies (El-Banna & Winkelmann, 1998, Loper & Gross, 2007).

One of the most interesting findings was that semi-purified extracts of *B. ambifaria* AMMD containing enacyloxin produced an inhibitory affect at low concentrations (15.6 μg ml^{-1}) on Gram-negative bacteria such as *E. coli, B. multivorans* and *A. baumanni.* Enacyloxin IIa isolated from *B. ambifaria* AMMD and BCF inhibited *C. albicans*. However, this does not support the previous studies of Watanabe, *et al*., (1982, 1994) as they reported that Enacyloxin IIa did inhibit both Gram-positive and Gram-negative bacteria but could not inhibit *C. albicans*. This might be because of the concentration that they used was lower. Mahenthiralingam, *et al*., (2011) found that Enacyloxin IIa at high concentration was active against *C. albicans*, but that below 100 μg ml^{-1} it was not significantly anti-candidal.

Bactobolin isolated from *B. ambifaria* BCF and Mex-5 showed a strong ability to inhibit *C. albicans* and other fungi belonging to basidiomycetes. However, this result has not previously been described.

4.3 Conclusion

The following conclusions were reached:

- Extraction efficiency of the Amberlite XAD-16 resin followed by methanol elution proved to be the most robust and simplest method for extracting the bioactive compounds at high purity from *Burkholderia*.

- TLC combined with a bioautography assay is a rapid and accurate method for localization of antimicrobial activity on a chromatogram. It offers the ability to obtain a profile of *Burkholderia* antimicrobial secondary metabolite patterns to study the presence and the diversity of antimicrobial agents of natural products.

- Ethyl acetate, methanol and water (20:1:0.5), Normal silica gel TLC plates, UV light (366 nm) and 15 min of aeration were the best conditions to separate and visualise *Burkholderia* secondary metabolites.

- The inoculum size of the microorganisms for bioautography must be specifically adapted for use with different test organisms.

- Pre-incubation of overlaid TLC plates for at least 3 h at room temperature was critical for improving the detection of bioactivity to allow an active metabolite to diffuse.

- The TLC-bioautography and GC-MS data showed that *Burkholderia* are potentially a very large reservoir of known and novel antimicrobial agents, especially with those exhibiting antifungal activity. Many of these compounds were discovered for the first time in this group of bacteria.

- The *B. ambifaria* AMMD crude resin extract comprised five main antimicrobial compounds. The following were detected previously in other *Burkholderia* sp: (1) polyethylene glycol (2) quinolines group (4-hydroxyl-3-methyl-2-alkyl-4-quinoline, 4-hydroxyl-3-ethyl-4-quinoline and other HMAQ) and (3) pyrrolnitrin.

- *B. ambifaria* BCF produced 4 active bands which exhibited nearly the same profile as *B. ambifaria* AMMD. The following were detected: (1) quinolines group; (2) pyrrolnitrin; and (3) Enacyloxin IIa. *B. ambifaria* BCF produced highly active

metabolites which were identified as a mixture of bactobolin A, B, D and a new bactobolin containing Cl_2.

- *B. ambifaria* KW and Mex-5 produced more than 6 active bands containing at least 10 detectable but unknown active compounds and were presumed to be unknown antifungal agents.

- *B. ambifaria* Mex-5 produced highly active metabolites which were identified as a mixture of new bactobolin and were different to those obtained from the BCF strain. Bactobolin C was the major compound detected in Mex-5, but bactobolin D and B were not detected**.**

- Pyrrolnitrin, a known antibiotic previously found in *Burkholderia* and *Pseudomonas* was detected in 60% of tested *Burkholderia,* using classic comparative TLC and pure pyrrolnitrin (Sigma). Pyrrolnitrin showed a strong effect on *C. albicans* and basidiomycetes. It was also active against Gram-positive bacteria.

- Novel unknown peptide antibiotics were detected in *B. ambifaria* AMMD, BCF, KW and Mex-5.

- Semi-purified extracts of *B. ambifaria* AMMD (R*f* 0.60 ± 0.03) containing enacyloxin IIa produced an inhibitory affect at a low concentration (15.6 μg ml^{-1}) against *E. coli, B. multivorans* and *A. baumannii.*

- Bactobolin isolated from *B. ambifaria* BCF and Mex-5 showed a strong ability to inhibit *C. albicans* and other fungi belonging to basidiomycetes.

CHAPTER FIVE

OPTIMIZATION OF GROWTH CONDITIONS TO IMPROVE ANTIFUNGAL ACTIVITY OF *Burkholderia*

5.1 Introduction

The biosynthesis of natural products by organisms is affected by biotic and abiotic factors. In the laboratory, the components of synthetic media and different factors such as pH, temperature and incubation period alter antibiotic biosynthesis in many producer strains. Understanding these production conditions in the laboratory will provide an insight into when antimicrobial agents are produced in nature.

Carbon, nitrogen and phosphate are the major requirements for the growth and metabolic activities of microorganisms. Nitrogen is one of the major requirements because it is required for the synthesis of cell components and functional proteins. It is well-known that the different kinds of nitrogen source greatly influence microbial secondary metabolite production. It is interesting that inorganic phosphate controls the synthesis of a large number of antibiotics that belong to different classes (Martin, 2004). However, most of the antibiotics were repressed by high concentrations of inorganic phosphate (Kishimoto*, et al.*, 1996). The pH of growth media also exerts an effect on the production of antimicrobial agents. The pH may change the ionizing state of important vital molecules such as proteins and enzymes. The production of antimicrobial agents is dependent on many enzyme catalysed reactions, so pH is one of the most important physical factors affecting anti-candidal production and activity.

Previous research in our laboratory had shown that *Burkholderia* bacteria grew well on a chemically-defined minimal medium, named Basal Salts Medium (BSM; section 2.1). The production of antifungal agents by *Burkholderia* was shown to occur when they were grown on BSM with glycerol as a carbon source (Chapter 3 and 4). The objective of this

chapter was to improve the composition of BSM to enhance antimicrobial production by *Burkholderia.* Since BSM was a defined minimal salts medium it was possible to change the composition of its ingredients and its pH, and detect the effects of a target nutrient on antimicrobial production**.** A general hypothesis for this chapter was **''chemical and physical factors can influence anti-candidal production and activity of *Burkholderia*''**, and the specific objectives of this chapter were:

(i) To examine the effects of six different factors: carbon, nitrogen, phosphate, pH, incubation time and temperature on the activity of anti-candidal agents of *Burkholderia*.

(ii) To create an optimal medium to increase anti-candidal activity of *Burkholderia* metabolite.

5.2 Materials and Methods

5.2.1 Bacterial and fungal strain maintenance and revival

The well defined antifungal control strains examined in previous chapters, *B. ambifaria* AMMD, BCF, KC311, *B. vietnamiensis* JW13 and two reference fungi, *C. albicans* Sc5314 and *B. adusta* BK1, were used to assess physical and chemical conditions affecting the growth and production of antifungal agents. The effect of various factors on the production of anti-candidal metabolites was compared to the standard Basal Salts Medium (BSM; see sections 2.1). The maintenance and storage conditions of these isolates were as described previously (sections 2.2.1 and 2.2.2).

5.2.2 Effects of different growth factors on anti-candidal activity of *Burkholderia*

To determine the best conditions for both fungal growth and anti-candidal activity of *Burkholderia* metabolites (section 2.7), different concentrations and combinations of chemical factors such as carbon, nitrogen and phosphate were altered in the BSM growth medium. In addition, the three physical factors: hydrogen ion concentration, temperature and period of incubation were also examined. The effect of hydrogen ion concentration on the growth abilities of *B. adusta* and *C. albicans* was also examined. All experiments were carried out at 30°C for three days unless stated, with three replicates of each treatment.

5.2.3 Statistics

One and two-way analysis of variance and the General Linear Model were used to test for differences between treatments and strains.

5.3 Results

5.3.1 The effect of carbon source on anti-candidal activity of *Burkholderia*

5.3.1.1 Effect of carbon sources

All tested isolates showed a significant difference ($P \leq 0.001$) in production of anti-candidal agents (Figure 5.1). Glycerol, fructose, galactose, glucose and succinate significantly ($P \leq 0.001$) increased the activity of *Burkholderia* anti-candidal agents . *B. ambifaria* Kc311was the strongest producer among tested isolates (36.8 mm). However, there was very low anti-candidal activity on lactose, starch and maltose. *B. ambifaria* BCF, KC311 and *B. vietnamiensis* JW13, showed similar responses to the carbon sources tested. Their anti-candidal activity was greatest after growth on glycerol followed by fructose and succinate (Figure 5.1). However, no anti-candidal activity of *B. ambifaria* BCF was seen with sucrose as a sole carbon source; *B. vietnamiensis* JW13 did not produce any activity when grown on lactose as a sole of carbon source. The anti-candidal activity of *B. ambifaria* AMMD was greatest when grown on succinate, glycerol, and ribose. However, there was a weak affect of AMMD metabolites on candidal growth when grown on arabinose, lactose and maltose, and there was no anti-candidal activity when starch was used as a sole carbon source (Figure 5.1).

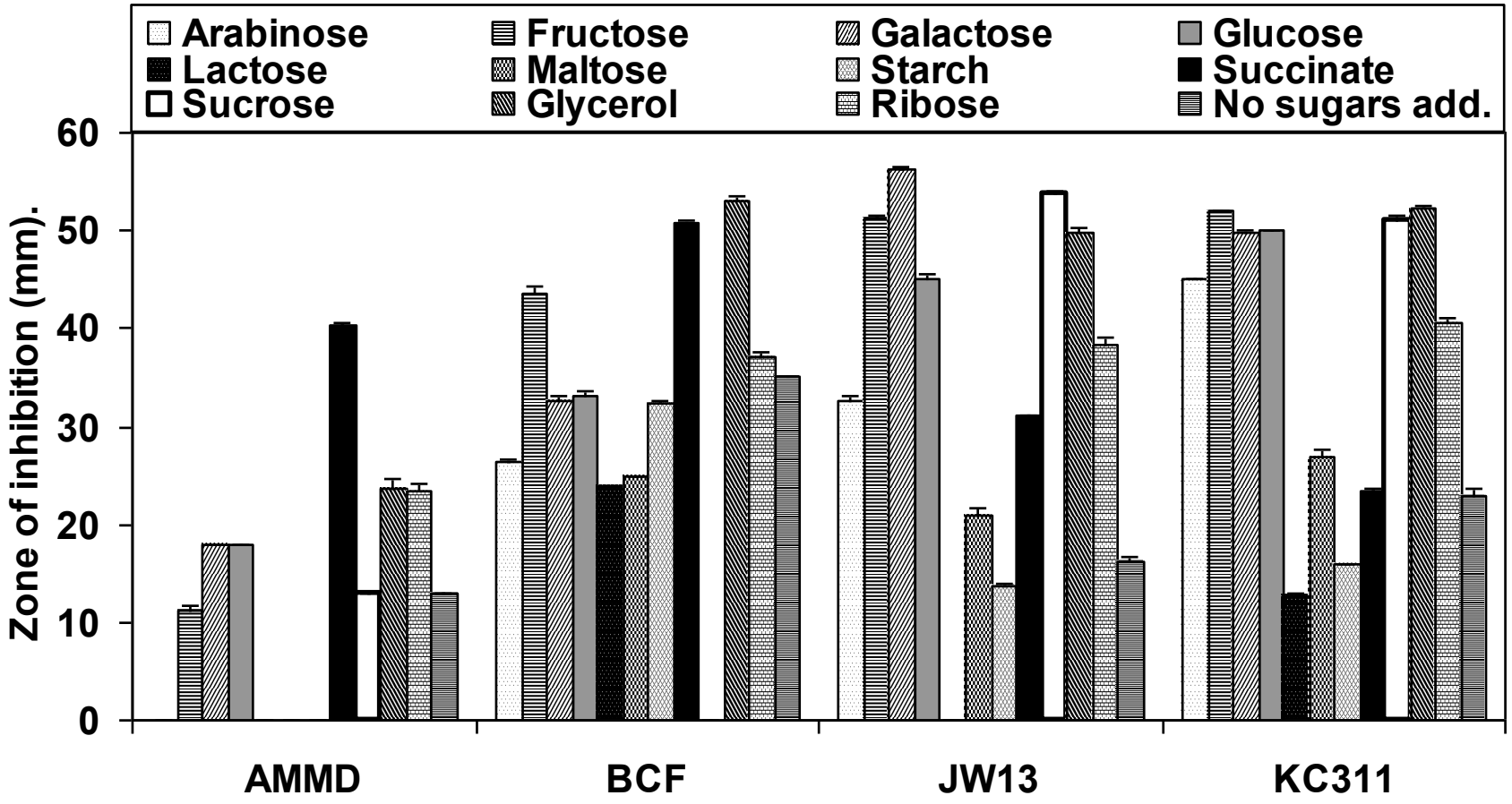


Figure 5.1 Effect of 12 carbon sources on anti-candidal activity of *B. ambifaria* AMMD, BCF, KC311 and *B. vietnamiensis* JW13.

Error bars are not visible in some cases where values approach 0; when no error bars are shown they fall within the size of the symbol. Data are presented as the means of three replicates with standard errors of the means.

5.3.1.2 The effect of different concentrations of glycerol and succinate on antifungal activity

All four control strains *B. ambifaria* AMMD, BCF, KC311 and *B. vietnamiensis* JW13 that were tested, could grow well on either carbon source (glycerol or succinate) with or without supplements such as CAS amino acids or yeast extracts (Figure 5.4 a, b, c, d). However, they lost the ability to grow and produce active anti-candidal agents when the medium was not supplemented with CAS amino acids and yeast extract.

In general, there were significant differences between different carbon source concentrations ($P \leq 0.05$). The optimum carbon concentrations (Figure 5.5) were 40 mM and 20 mM (30.7 and 29.13 mm respectively). In terms of media supplementation for either a glycerol or succinate carbon source, BSM with CAS amino acids and complete BSM medium with both CAS amino acids and yeast extract produced the largest zones of inhibition against *C. albicans* (Figure 5.6). Overall, production of anti-candidal agents on glycerol was better than using succinate (Figure 5.2).

B. ambifaria BCF and *B. vietnamiensis* JW13 showed the greatest activity against *C. albicans* with complete BSM medium supplemented with glycerol at 40 mM (63.37 and 49.7 mm respectively; Figure 5.2: b, d). *B. ambifaria* Kc311 exhibited the greatest activity with BSM supplemented with CAS amino acids and glycerol at 40 mM (51.30 mm, Figure 5.2, c). *B. ambifaria* AMMD showed significantly ($P \leq 0.05$) anti-candidal activity when glycerol was used as a sole carbon source at 40 and 20 mM (41.3 and 40.5 mm respectively; Figure 5.2, a). The optimal media combinations were BSM supplemented with CAS amino acids and glycerol (44.3 mm) and complete BSM medium supplemented with glycerol (41.3 mm).

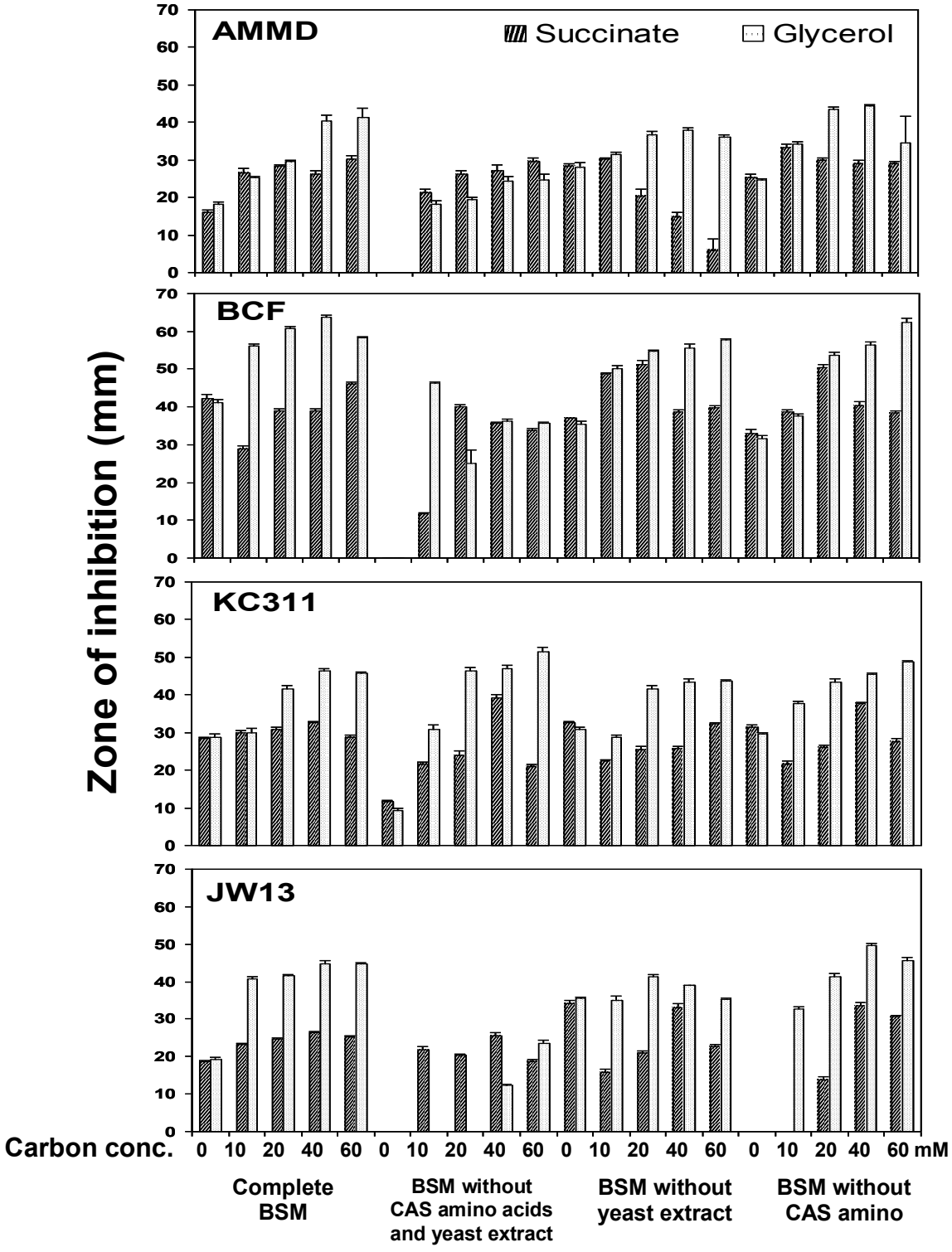


Figure 5.2 Effect of glycerol and succinate at different concentrations (0 - 60 mM) on anti-candidal activity of *B. ambifaria* AMMD, BCF, KC311 and *B. vietnamiensis* JW13, using BSM medium with different combinations.

Error bars are not visible in some cases where values approach 0; when no error bars are shown they fall within the size of the symbol. Data are presented as the means of three replicates with standard errors of the means.

5.3.2 The effect of nitrogen

5.3.2.1 The effect of nitrogen sources on activity of anti-candidal agents

B. ambifaria Kc311 and BCF showed the greatest effect (44.5 and 42 mm, respectively) against *C. albicans* (Figure 5.3). *B. ambifaria* BCF and AMMD showed similar responses with different nitrogen sources used; the production of active anti-candidal agents by these strains was greatest with yeast extract (55.3 and 27.3 mm, respectively), followed by ammonium phosphate (52.3 and 24.67 mm, respectively). The anti-candidal activity of *B. ambifaria* KC311 was optimum with ammonium phosphate (55.3 mm) and ammonium chloride (51.3 mm). However, *B. vietnamiensis* JW13 showed the most anti-candidal activity with ammonium chloride (55.3 mm) and CAS amino acids (52.67 mm). However, JW13 did not grow on BSM-G supplemented with sodium nitrite, potassium nitrate or ammonium nitrate as sole nitrogen sources (Figure 5.3).

Sodium nitrate totally suppressed the production of anti-candidal agents by all tested *Burkholderia*. Ammonium nitrate and potassium nitrate were significantly ($P \leq 0.01$) poorer at stimulating anti-candidal activity than the other nitrogen sources tested. There was good anti-candidal activity when ammonium phosphate, CAS amino acids, ammonium chloride, peptone and yeast extract were added to the medium (45.5, 44, 43.5, 43 and 41 mm respectively). However, there were no significant differences between these nitrogen sources (Figure 5.3).

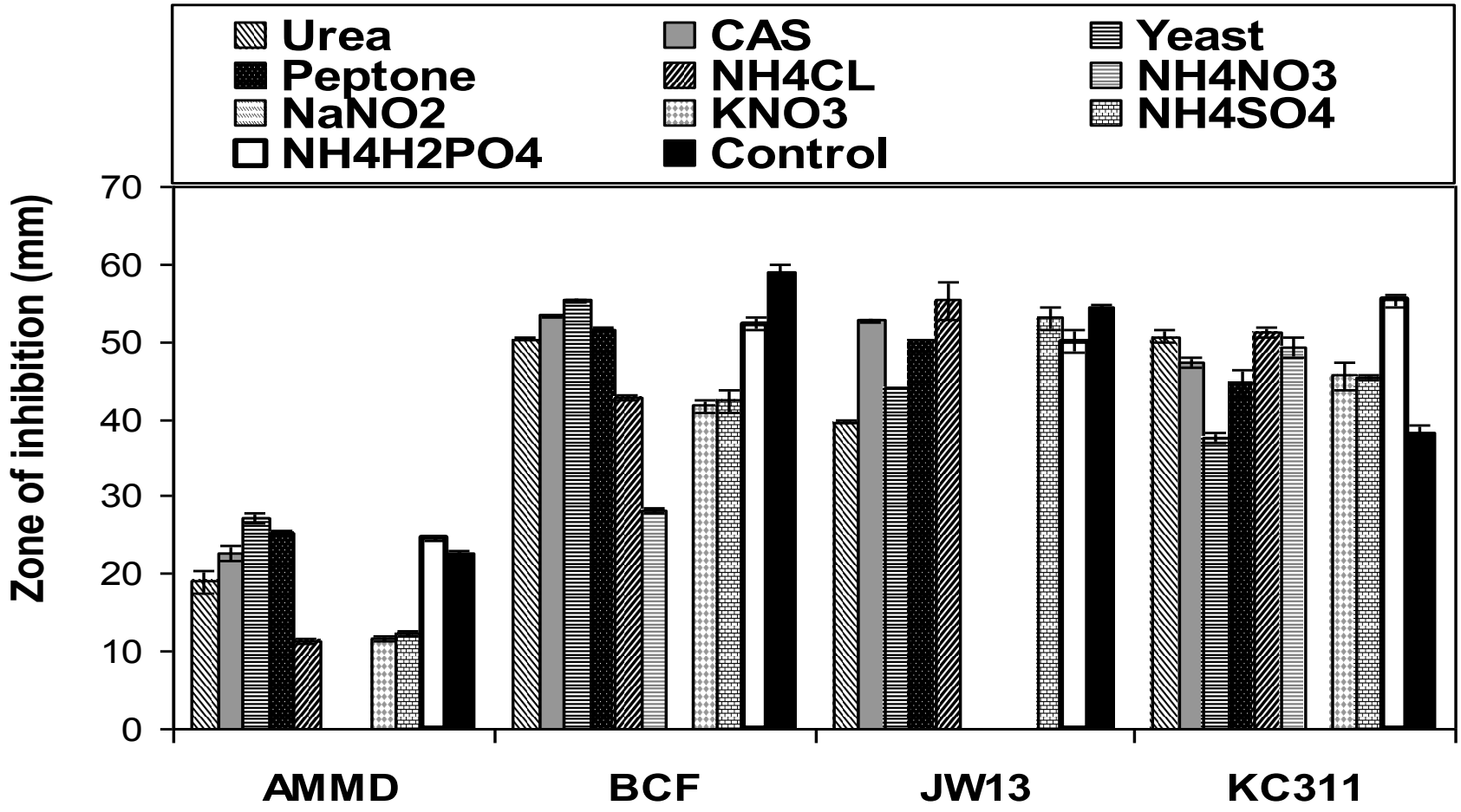


Figure 5.3 Effect of different nitrogen sources on anti-candidal activity of *B. ambifaria* AMMD, BCF, KC311 and *B. vietnamiensis* JW13.

Error bars are not visible in some cases where values approach 0; when no error bars are shown they fall within the size of the symbol. Data are presented as the mean of three replicates with standard errors of the mean.

5.3.2.2 The effect of ammonium phosphate in different combinations with BSM-G on anti-candidal activity of *Burkholderia*

Anti-candidal activity was influenced by different combinations of BSM-G (Figure 5.4). The optimal combinations were ammonium phosphate, yeast extract and CAS amino acids, and ammonium chloride, yeast extract and CAS amino acids (43, 41.6 mm respectively). Moreover, there were highly significant differences between *Burkholderia* isolates ($P \leq 0.001$). *B. ambifaria* Jw13 and BCF were the best in the production of active anti-candidal agents under different conditions (Figure 5.4).

5.3.2.3 The effect of different concentrations of yeast extract at different pHs on anti-candidal activity

The effect of different concentrations of yeast extract at two different pHs (6 and 7) on anti-candidal activity was assessed on BSM-G medium. No significant difference in the effect of yeast extract at different pHs was observed for the production of anti-candidal agents ($P > 0.05$). 2 g of yeast extract at pH 6 was the optimal concentration to increase the production of anti-candidal agents (Figure 5.5). *B. vietnamiensis* JW13 produced good anti-candidal activity at different concentrations of yeast extract: 0.5, 1.0 and 2.0 gl^{-1} at pH 6 (53, 50.67 and 50.67 mm respectively). The highest anti-candidal activity for *B. ambifaria* AMMD was obtained by addition of 2 gl^{-1} yeast extract at pH 6 (29.67 mm). *B. ambifaria* BCF showed greatest activity (51.00 mm) at 2 gl^{-1} yeast extract at pH 7. The maximum anti-candidal activity of *B. ambifaria* KC311 was obtained by addition of either 1 or 2 gl^{-1} yeast extract at pH 6 (70.0 and 68.67 mm respectively). However, there were significant differences ($P \leq 0.05$) between *Burkholderia* isolates (Figure 5.5).

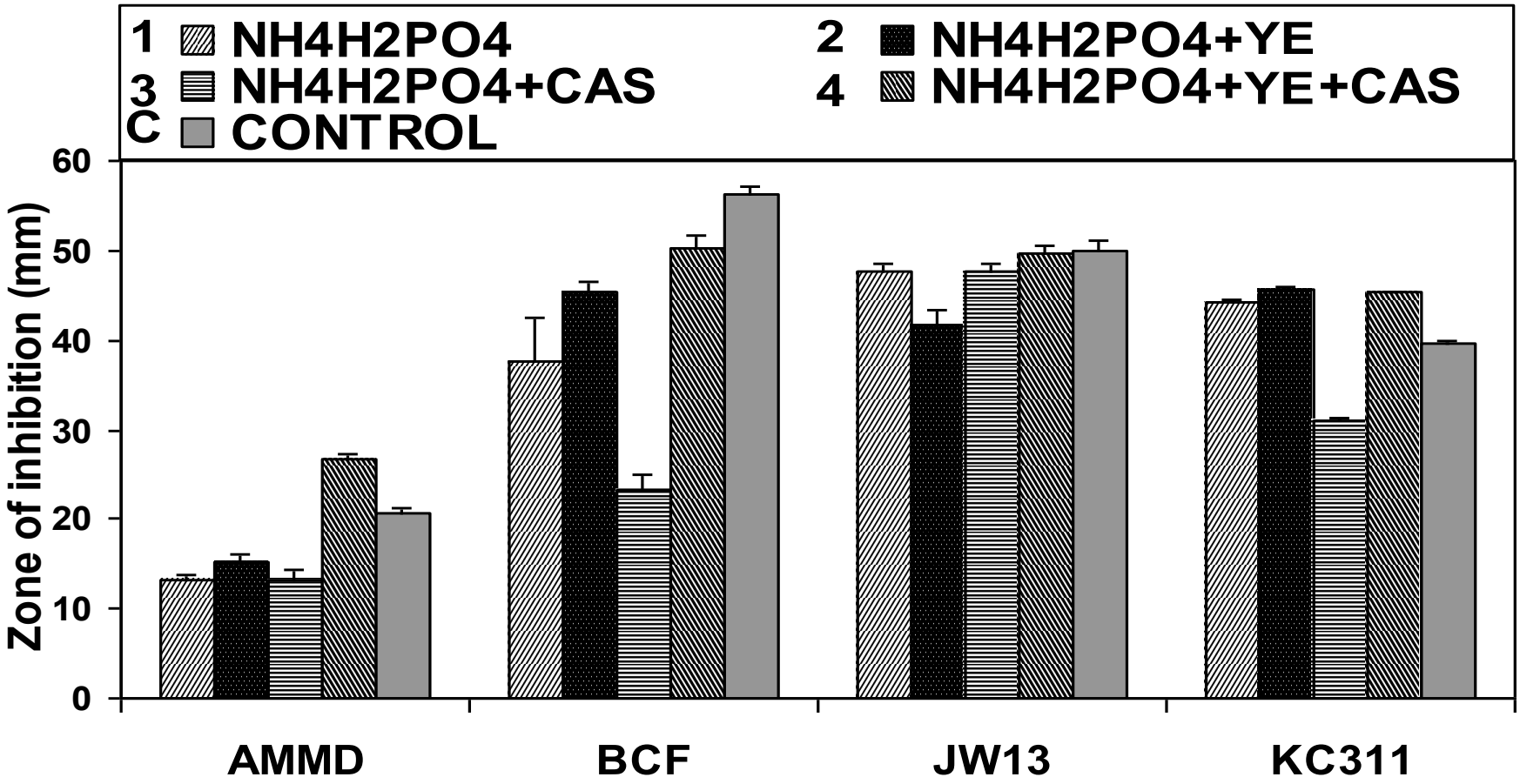


Figure 5.4 Effect of ammonium phosphate alone and with different combinations of BSM-G on anti-candidal activity of *B. ambifaria* AMMD, BCF, KC311 and *B. vietnamiensis* JW13.

C, normal BSM-G; 1, BSM-G supplemented with ammonium phosphate (NH4H2PO4) without yeast extract (YE) and CAS amino acids (CAS); 2, BSM-G supplemented with ammonium phosphate and yeast extract; 3, BSM-G supplemented with ammonium phosphate and CAS amino acids; 4, BSM-G supplemented with ammonium phosphate, yeast extract and CAS amino acids. Data are presented as the mean of three replicates with standard error of the mean.

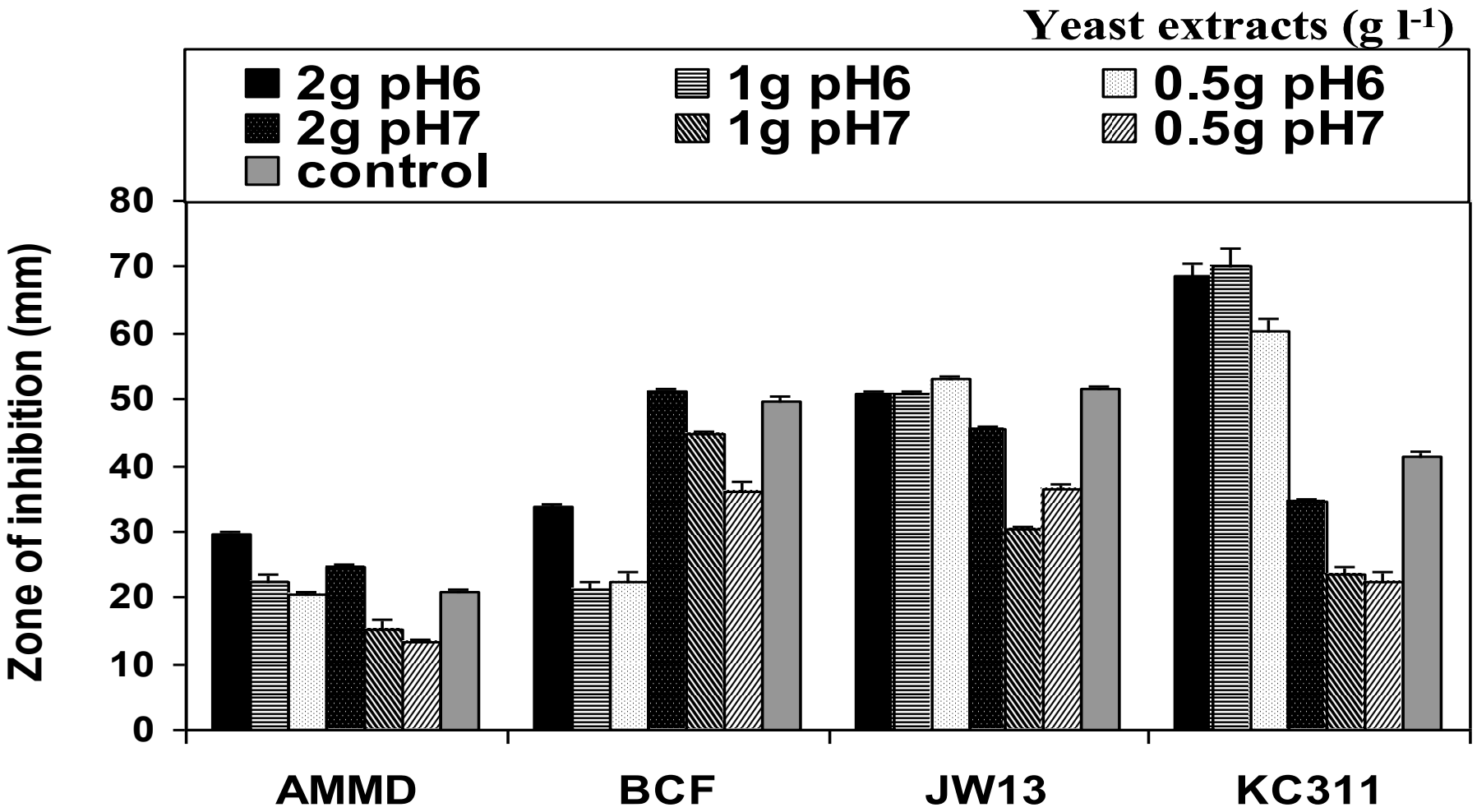


Figure 5.5 Effect of yeast extract at different concentrations (g l^{-1}) and different pHs (6 and 7) in BSM-G on anti-candidal activity of *B. ambifaria* AMMD, BCF, KC311 and *B. vietnamiensis* JW13.

When no error bars are shown they fall within the size of the symbol. Data are presented as the means of three replicates with standard errors of the means.

5.3.3 The effect of different phosphate sources and concentration on the anti-candidal activity of *Burkholderia*

Different phosphate sources at different concentrations did not significantly ($P > 0.05$) affect anti-candidal activity of the four *Burkholderia* strains. In general, lower concentrations of phosphate (10 mM or less) gave better anti-candidal activity than higher concentrations (40 mM or greater). In general, sodium phosphate at 10 mM enhanced the production of anti-candidal agents for all strains (Figure 5.6).

The results showed that there were highly significant differences ($P \leq 0.05$) between *Burkholderia* isolates on anti-candidal activity and the best producer was *B. ambifaria* BCF. *B. ambifaria* BCF produced the greatest anti-candidal activity at 80 mM sodium phosphate (45.00 mm), which was only slightly above that seen at 10 mM sodium phosphate. The activity of anti-candidal activity of *B. ambifaria* AMMD and KC311 was best with sodium phosphate at 10 mM, producing inhibition zones of 29.3 and 48.3 mm respectively. Anti-candidal activity of *B. vietnamiensis* was greatest with the addition of calcium phosphate at 80 mM, producing an inhibition zone of 45.0 mm (Figure 5.6).

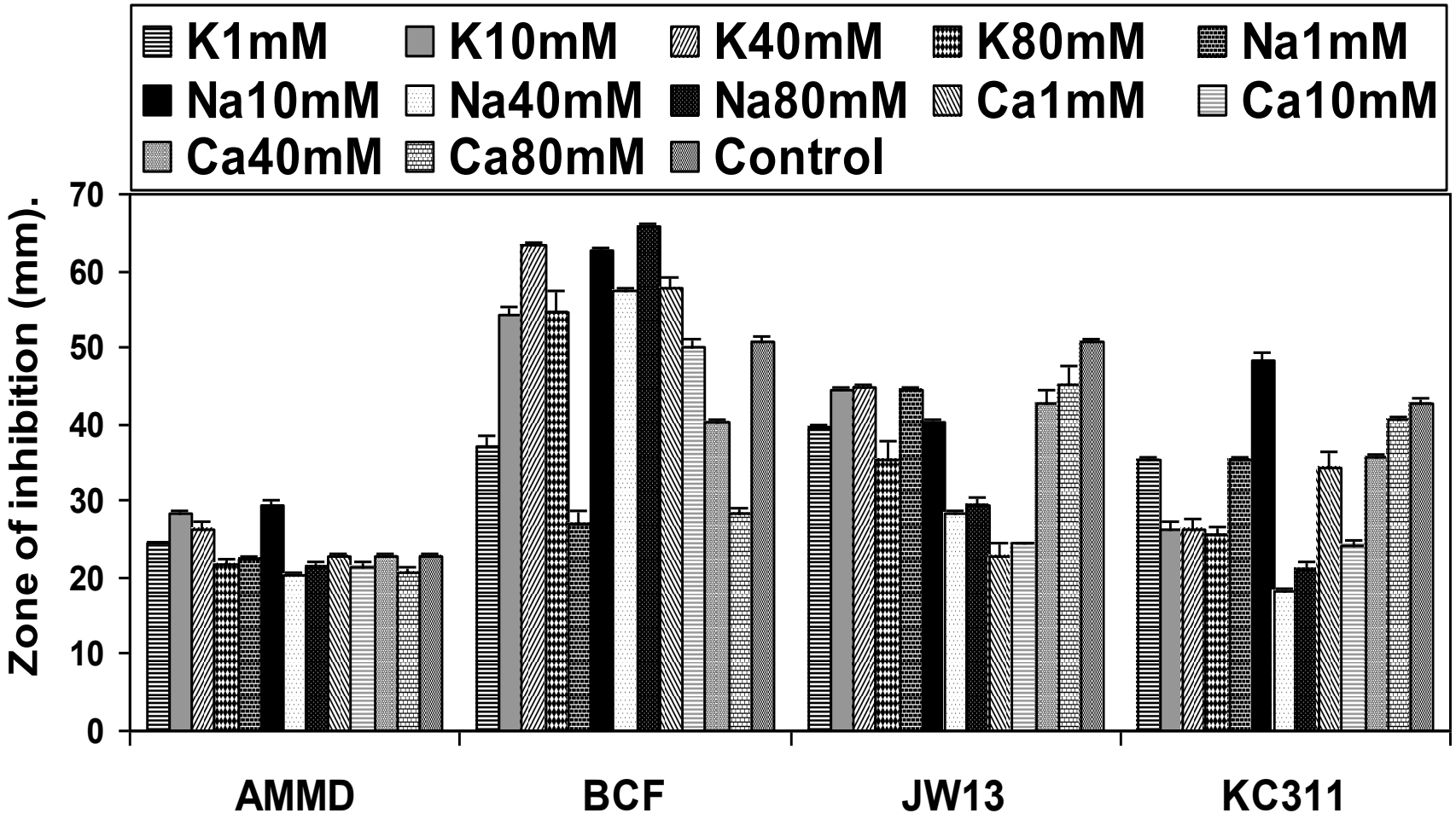


Figure 5.6 Effect of different phosphate sources at different concentrations of BSM-G on anti-candidal activity of *B. ambifaria* AMMD, BCF, KC311 and *B. vietnamiensis* JW13.

(K); Potassium phosphate, (Na); sodium phosphate and (Ca); calcium phosphate. When no error bars are shown they fall within the size of the symbol. Data are presented as the means of three replicates with standard errors of the means.

5.3.4 The effect of hydrogen ion concentration (pH)

5.3.4.1 The effect of pH on *C. albicans* and *B. adusta* BK1 growth rate

Prior to testing the production of antifungal metabolites by the *Burkholderia* strains, the growth of the *C. albicans* was tested at a range of pHs to ensure that it would grow normally in the overlay and hence still be able to act as a readout for antimicrobial activity. *C. albicans* was able to grow over a wide range of pHs (5 to 8); the best growth occurred when the pH ranged from 6.5–7.5 (Figure 5.7).

B. adusta BK1 was capable of growing on BSM-G over a wide range of pH (Figure 5.21), with the best growth occurring at pH 6 and 6.50 (extension rate 10.53 and 9.70 mm d^{-1} respectively). For all pHs tested, the *B. adusta* growth was sufficient to allow the mycelia to cover a 90 mm Petri dish of BSM-G medium within 6 days. The weakest growth of *B. adusta* where mycelial dense occurred was seen at the extremes of the pH range tested, 3 and 8, respectively (Figure 5.8).

Overall, both fungal species used to detect antifungal activity, *C. albicans* and *B. adusta* BK1, were capable at growth over a wide range of pH, demonstrating that bioassay detection of *Burkholderia* metabolites could be assayed at multiple the hydrogen ion levels.

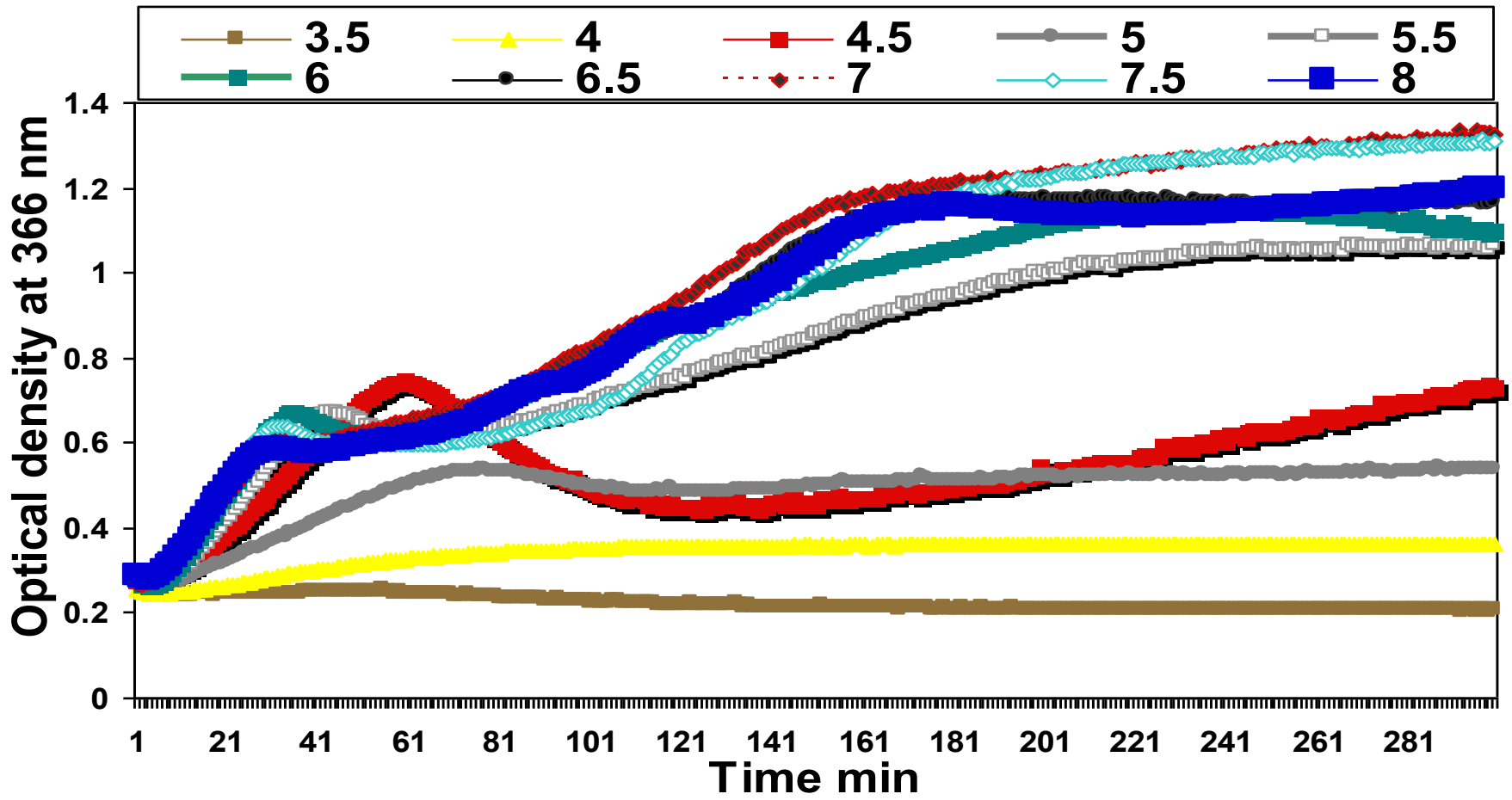


Figure 5.7 Effect of pHs (3.5-8) of BSM-G medium on *C. albicans* growth rate.

Data are presented as the means of three replicates with standard errors of the means. When there are no error bars are shown they fall within the size of the symbol.

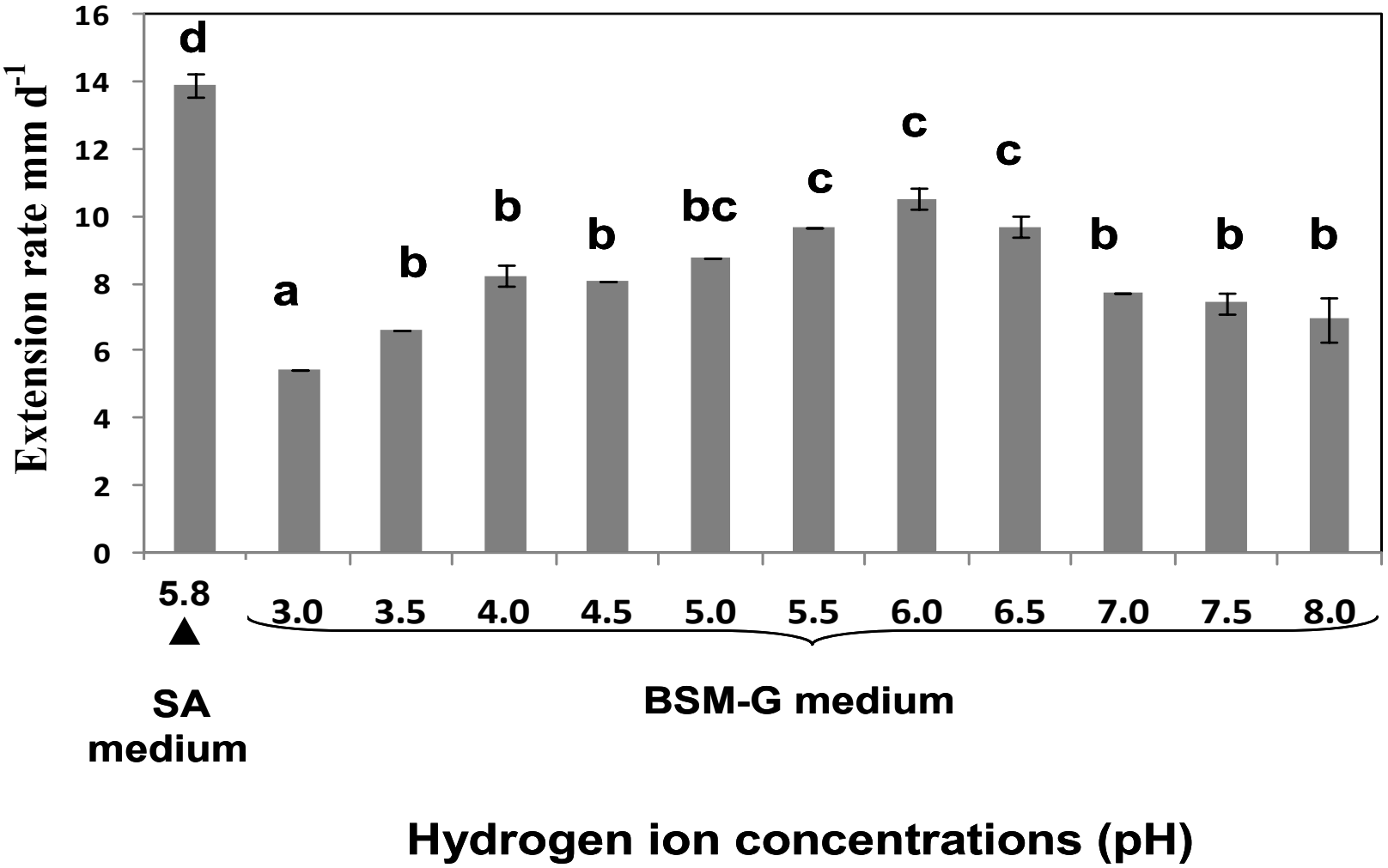


Figure 5.8 Effect of pHs (3-8) of BSM-G medium on *B. adusta* BK1 extension rate (mm d^{-1}).
SA: Sabouraud Agar at pH 5.8 was used as a control medium. BSM-G: Basal Salt Medium supplemented with glycerol. When there are no error bars are shown they fall within the size of the symbol. The same letter above bars indicates no significant difference (ANOVA; $P > 0.05$).

5.3.4.2 The effect of pH on anti-candidal activity of *B. ambifaria* AMMD, BCF, KC311 and *B. vietnamiensis* JW13

Three *Burkholderia* isolates, *B. ambifaria* AMMD, KC311 and *B. vietnamiensis* Jw13 preferred acidic conditions, around pH 6.0 to 6.5 where anti-candidal activity was greatest (Figure 5.22). The activity of *Burkholderia* anti-candidal agents was significantly affected depending on initial concentrations of hydrogen ion (Figure 5.9; $P \leq 0.001$). For example, at pH 6 the anti-candidal inhibition zones were 34.33, 58.67 and 60.67 mm, respectively, for these three strains. In contrast *B. ambifaria* BCF maintained a high level of anti-candidal activity after growth at pH 6.5 through to pH 8, while below pH 6.5 the activity of this strain was greatly diminished (Figure 5.9).

B. ambifaria AMMD grew and produced active anti-candidal agents at all tested pHs (3.5 - 8), although at the acidic pH of 4.5 and below this activity was less than 50% of that seen at the optimal pH of 6 for this strain. In contrast, no activity was seen for *B. ambifaria* BCF and KC311 below pH 4.5, and *B. vietnamiensis* did not produce anti-candidal agents below pH 4 (Figure 5.9).

5.3.5 The effect of temperature on anti-candidal activity of *B. ambifaria* AMMD, BCF, KC311 and *B. vietnamiensis* JW13

The results showed that the activity of anti-candidal agents steadily increased with an increase in temperature from 10^{o}C through to 35^{o}C, then decreased above this temperature. There were highly significant differences in anti-candidal activity between different temperatures ($P \leq 0.001$) and between *Burkholderia* isolates ($P \leq 0.05$) (Figure 5.10).

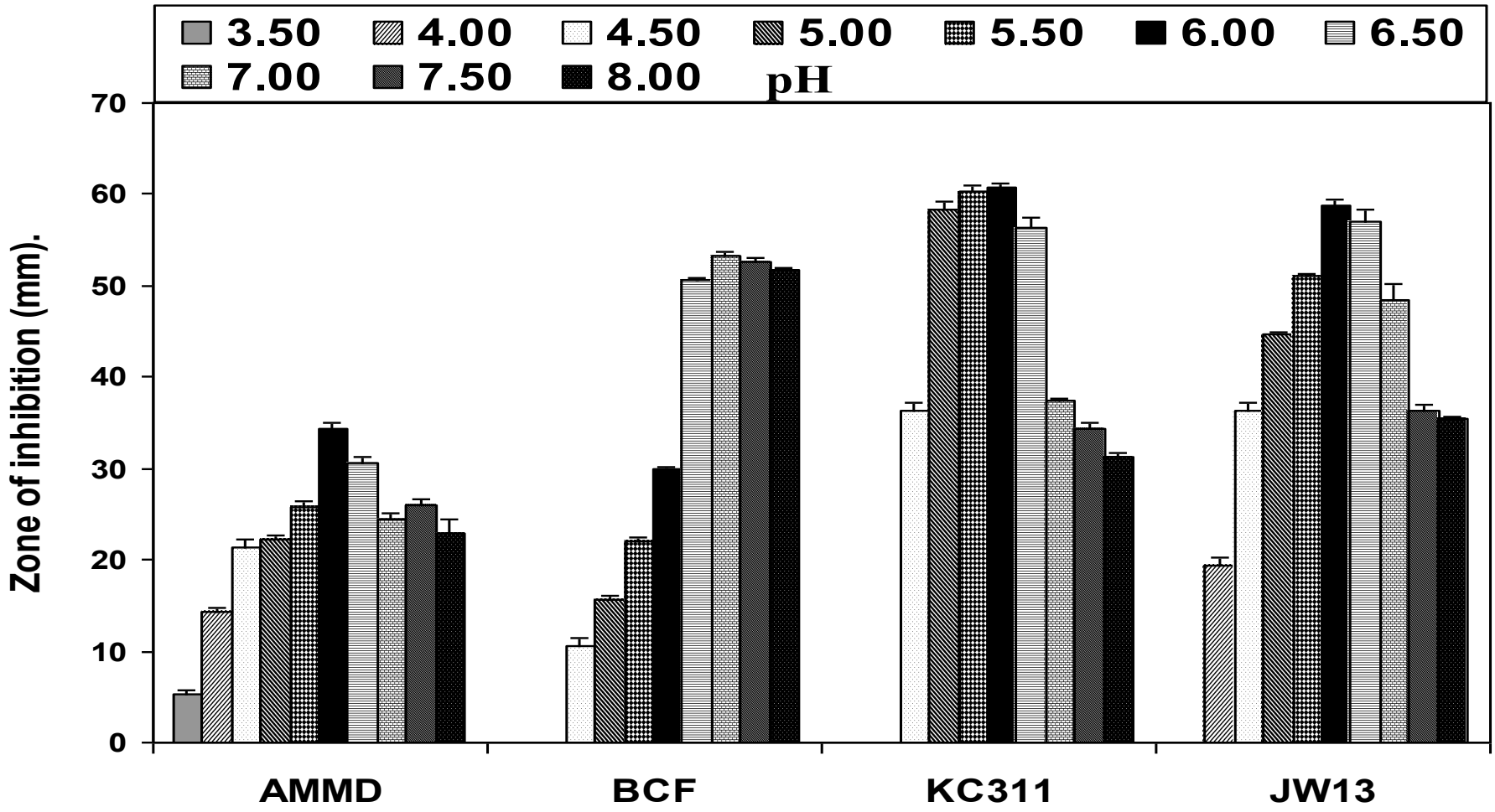


Figure 5.9 Effect of hydrogen ion concentration (3.5-8) of BSM-G on anti-candidal activity of *B. ambifaria* AMMD, BCF, KC311 and *B. vietnamiensis* JW13.

When there are no error bars are shown they fall within the size of the symbol. Data are presented as the mean of three replicates with standard error of the mean.

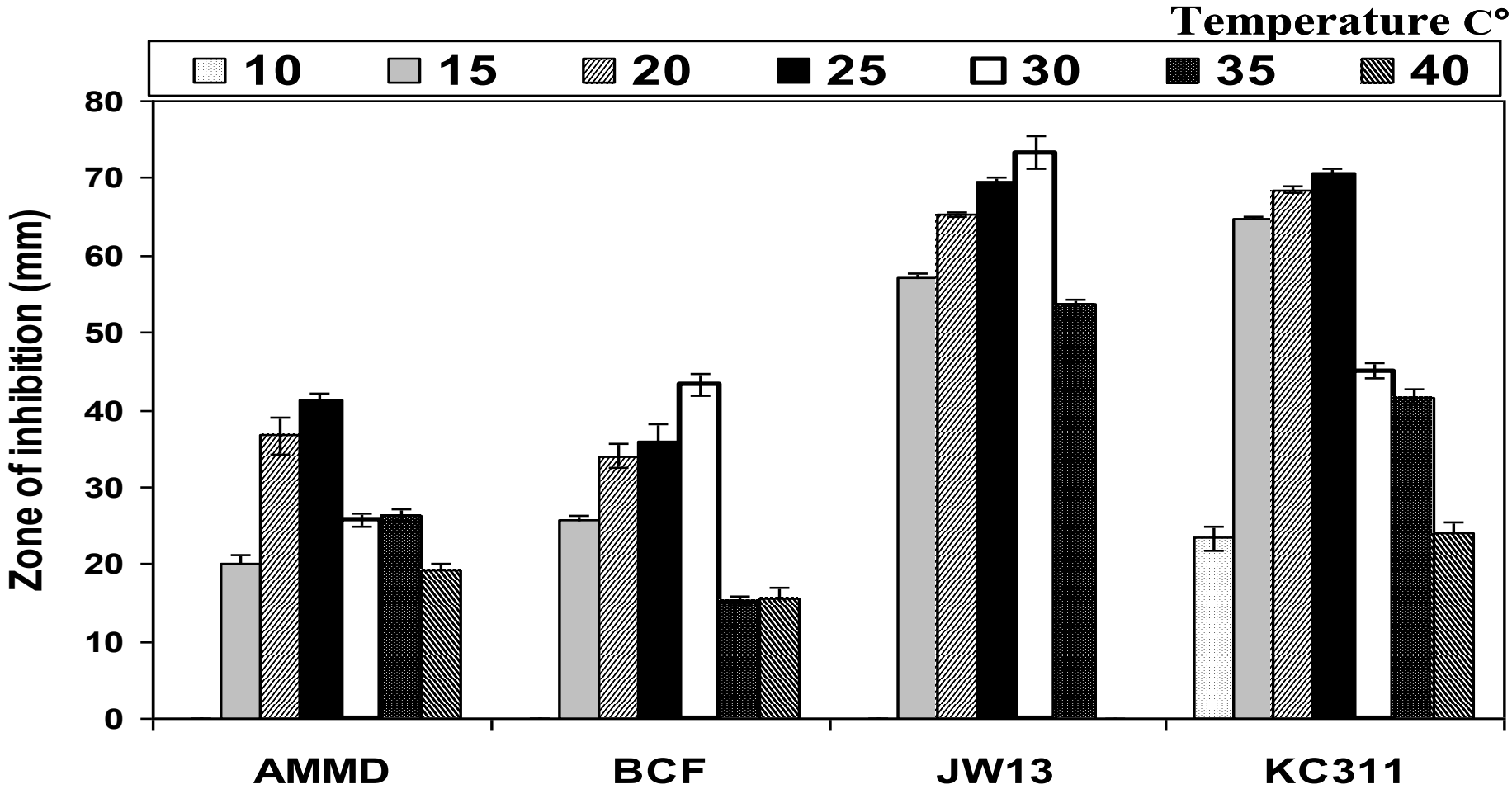


Figure 5.10 Effect of temperatures (10-40˚C) on anti-candidal activity of *B. ambifaria* AMMD, BCF, KC311 and *B. vietnamiensis* JW13.

When there are no error bars are shown they fall within the size of the symbol. Data are presented as the means of three replicates with standard errors of the means.

The anti-candidal activity of *B. ambifaria* AMMD and KC311 increased over time and reached maximum activity at 25°C (41.3 and 70.67 mm, respectively; Figure 5.27). *B. ambifaria* BCF and *B. vietnamiensis* JW13 metabolites reached their maximum activities at 30°C (43.3 and 73.3 mm respectively). *B. ambifaria* AMMD, BCF, and *B. vietnamiensis* JW13 metabolites did not show anti-candidal activity at 10°C, and *B. vietnamiensis* JW13 lost the ability to produce active anti-candidal agents above 40°C (Figure 5.10).

5.3.5 The effect of time of incubation on anti-candidal activity of *B. ambifaria* AMMD, BCF, KC311 and *B. vietnamiensis* JW13

The activity of anti-candidal agents steadily increased over time (Figure 5.11). Typically, 168 h was the best length of time, producing the maximum anti-candidal activity with highly significant differences ($P \leq 0.001$). Moreover, all isolates showed significant differences ($P \leq 0.05$) in the production of active anti-candidal agents at different times of incubation. Anti-candidal activity after growth on BSM-G medium was demonstrated by all four tested strains within 24 h (Figure 5.11). The anti-candidal activity of *B. ambifaria* BCF and *B. vietnamiensis* JW13 metabolites increased over time and achieved the maximum by 120 h of incubation (46.0 and 80.0 mm respectively). However, *B. ambifaria* AMMD and KC311 reached the maximum capacity of activity at 168 h of incubation (46.0 and 80.0 mm respectively).

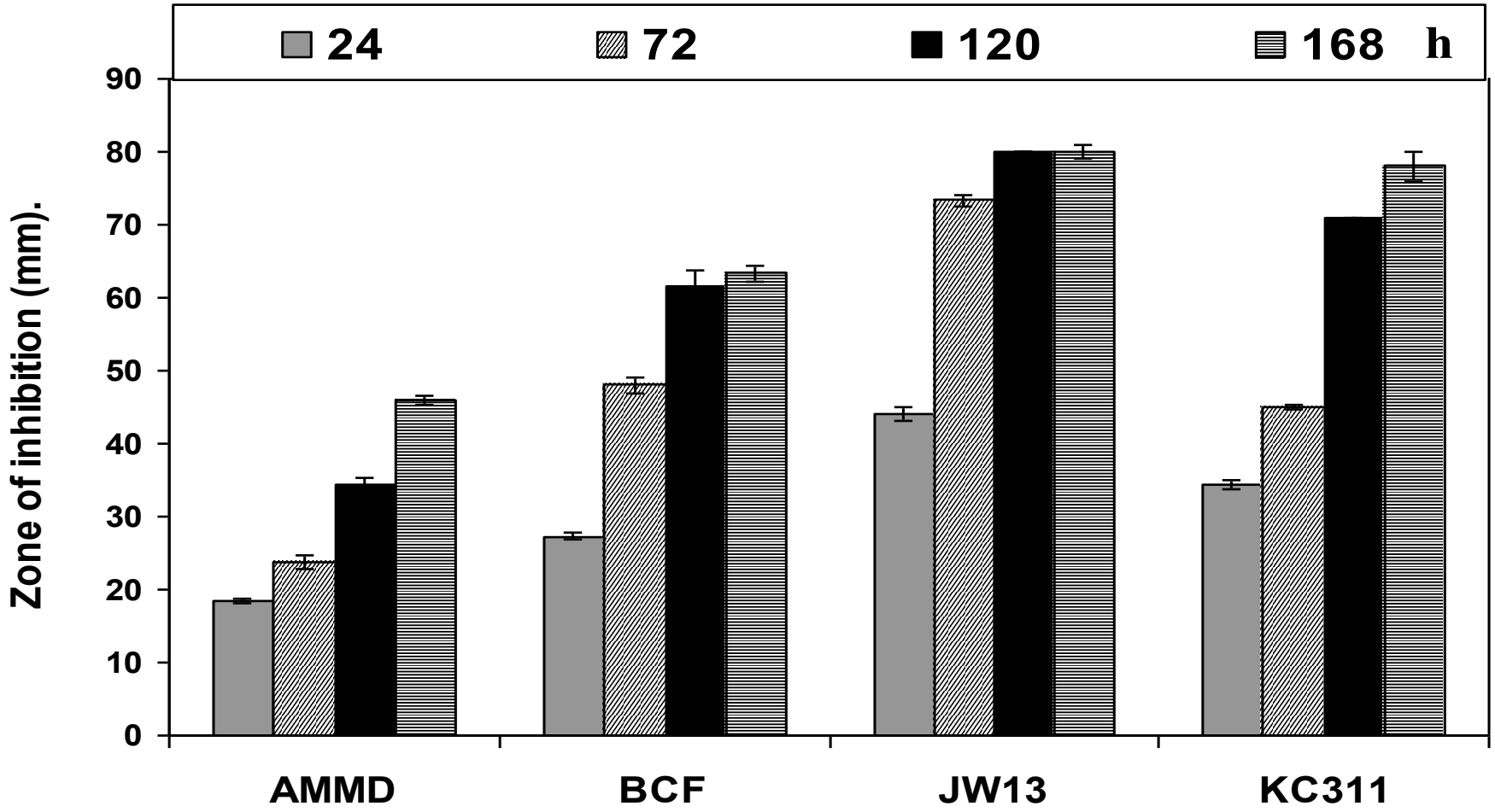


Figure 5.11 Effect of time of incubation (24-168 h) on anti-candidal activity of *B. ambifaria* AMMD, BCF, KC311 and *B. vietnamiensis* JW13.

When there are no error bars are shown they fall within the size of the symbol. Data are presented as the means of three replicates with standard errors of the means.

5.3.6 The effect of different formulae of basal salts medium (BSM) on anti-candidal activity of *B. ambifaria* AMMD metabolites

The main purpose of the investigations described above was to enable the best media combinations and conditions to be selected for optimized production of anti-candidal activity in the *Burkholderia* strains. Using the data of current study, combinations of 12 different media were tested on *B. ambifaria* AMMD, all using glycerol as the carbon source (Figure 5.12). Medium composition influenced the activity of anti-candidal agents produced by *B. ambifaria* AMMD ($P \leq 0.001$). The BSM4.6 medium (BSM supplemented with ammonium phosphate at 0.037 M and 0.05 g yeast extract as nitrogen sources and 20 mM HEPES to maintain the pH at 6) was superior in supporting the production of anti-candidal agents (38.67 mm). The next best medium was BSM5.6 (BSM supplemented with ammonium phosphate at 0.037 mM and 0.05 g yeast extract as nitrogen source and 0.02 mM phosphate buffer to maintain the pH at 6). The third best medium was BSM0.6 (normal BSM with 0.02 mM phosphate buffer to maintain the pH at 6; 36.33 mm). For these three anti-candidal activity promoting media there were no significant differences in the zones of inhibition observed. The BSM2.7 (BSM supplemented with 2 g yeast extract as nitrogen source and 0.02 mM HEPES to maintain the pH at 7) showed the lowest anti-candidal activity (24.67 mm) compared with all the other treatments (Figure 5.12).

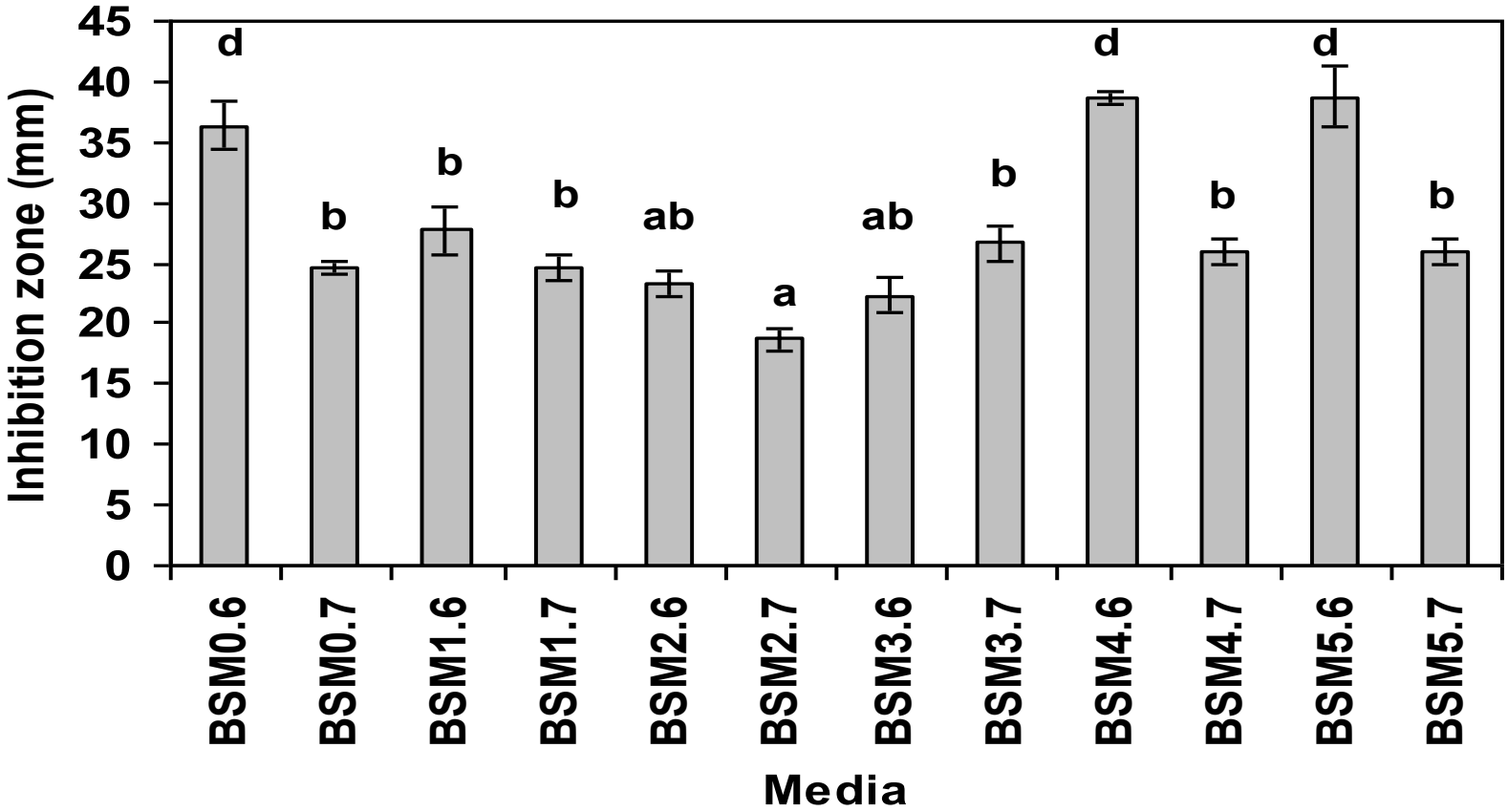


Figure 5.12 Effect of different formulae of BSM medium on anti-candidal activity of *B. ambifaria* AMMD.

BSM0.6, Normal BSM with 0.020 mM phosphate buffer to maintain the pH at 6; **BSM0.7**, Normal BSM with phosphate buffer to maintain the pH at 7; **BSM1.6**, BSM supplemented with 2 g yeast extract as nitrogen source and 0.020 mM phosphate buffer to maintain the pH at 6; **BSM1.7,** BSM supplemented with 2 g yeast extract as nitrogen source and 0.020 mM phosphate buffer to maintain the pH at 7; **BSM2.6**, BSM supplemented with 2 g yeast extract as nitrogen source and 20 mM HEPES to maintain the pH at 6; **BSM2.7,** BSM supplemented with 2 g yeast extract as nitrogen source and 20 mM HEPES to maintain the pH at 7; **BSM3.6**, BSM supplemented with 2 g yeast extract as nitrogen source and 0.025 mM of sodium phosphate to maintain the pH at 6; **BSM3.7,** BSM supplemented with 2 g yeast extract as nitrogen source and 0.025 mM of sodium phosphate to maintain the pH at 7; **BSM4.6**, BSM supplemented with ammonium phosphate at 0.037 mM and 0.05 g yeast as nitrogen source and 20 mM HEPES to maintain the pH at 6; **BSM4.7**, BSM supplemented with ammonium phosphate at 0.037 mM and 0.05 g yeast as nitrogen source and 20 mM HEPES to maintain the pH at 7; **BSM5.6**, BSM supplemented with ammonium phosphate at 0.037 mM and 0.05g yeast as nitrogen source and 0.020 mM phosphate buffer to maintain the pH at 6; **BSM5.7**, BSM supplemented with ammonium phosphate at 0.037 mM and 0.05 g yeast as nitrogen source and 0.020 mM phosphate buffer to maintain the pH at 7. The same letter above bars indicates no significant difference (ANOVA; $P > 0.05$).

5.4 Discussion

The present study set out with the aim of assessing the importance of nutrients, including carbon, nitrogen and phosphate, as well as physical factors such as hydrogen ion concentration (pH) and temperature on the production and activity of *Burkholderia* anti-candidal metabolites. The production of antimicrobial metabolites is known to be influenced by media components and culture conditions, such as carbon, nitrogen, pH and temperature, each of which may vary from organism to organism in terms of the most optimal conditions (Iwai & Omura, 1982). Culture conditions such as carbon, nitrogen and hydrogen ion concentration were found to affect the production of antifungal metabolites by *Burkholderia* (Kadir*, et al.*, 2008). El-Banna and Winkelmann (1998) reported that by varying the conditions under which *B. cepacia* is grown, the activity of the pyrrolnitrin antibiotic could be changed.

The current study found that the production of anti-candidal substances of *B. ambifaria* AMMD, BCF and KC311 and *B. vietnamiensis* JW13 were strongly influenced by several chemical and physical factors. The effect of different carbon sources: glucose, glycerol, arabinose, fructose, galactose, sucrose, lactose, maltose, ribose, succinate and starch on the anti-candidal activity of *Burkholderia* metabolites, were examined. The production of anti-candidal agents of *B. ambifaria* AMMD, BCF and KC311 and *B. vietnamiensis* JW13 were acutely changed by the carbon source. The carbon source required for maximal anti-candidal activity was different among *Burkholderia* strains.

Glycerol strongly enhanced the anti-candidal activity of strains BCF, AMMD and KC311; whereas succinate and glycerol increased the anti-candidal activity of AMMD. This finding was consistent with that found by El-Banna and Winkelmann, (1998), and Bhattacharyya *et al*. (1998), who found glycerol was superior for antifungal production by *B*. *cepacia* NB-1 and *Streptomyces hygroscopicus* $D_{1.5}$ respectively. In contrast, the maximum anti-candidal activity of *B. vietnamiensis* JW13 was achieved after growth on galactose.

Arabinose, maltose and starch repressed the anti-candidal activity of AMMD. Moreover, Lactose stopped the anti-candidal activity of JW13 and AMMD. These findings suggest that each strain preferred a specific source of carbon to produce active anti-candidal agents. BSM medium without a carbon source showed minimal anti-candidal activity for all tested isolates. AMMD, BCF and JW13 were not able to grow on BSM medium without a carbon source, CAS amino acids and yeast extract. They were able to grow and produce anti-candidal agents on BSM medium without a carbon source but supplemented with at least one organic nitrogen source such as CAS amino acids, peptone or yeast extract, where both nitrogen and carbon were provided. This finding was expected and reflects the ability of these isolates to use organic nitrogen resources as a source of energy instead of carbon compounds. This finding was similar to results obtained by Gebreel *et al*. (2008), who reported that bacterial isolates belonging to *Bacillus*, *Corynebacterium* and *Cellulosimicrobium* were able to grow and produce antimicrobial agents with no carbon source.

One unanticipated finding was that KC311 was able to grow and produce anti-candidal agents on BSM medium without a carbon source or nitrogen source. This agreed with Duffy and Défago (1999), who found that *P. fluorescens* was able to grow and produce antibiotics and other metabolites even in the absence of a carbon source. This could be due to two reasons: firstly, the agar used in this study was a bacteriological grade which is quite pure, but might contain contaminating materials having enough nutrients to support minimal growth sufficient for some production of metabolites. The second reason might be that Kc311 was able to use dead cells of Kc311 inoculum as a nutrient to support minimal growth and production of anti-candidal agents.

A significant correlation was found between anti-candidal activity and concentrations of carbon sources. The anti-candidal activity increased when the concentration of succinate and glycerol increased up to 20 mM and 40 mM respectively. However, above these concentrations the activity decreased. Gupta and Kulkarni (2002) tested the effect of different concentrations of sugar (2, 5 and 7.5%) on the production of antifungal agents of *Thermomonospora* sp MTCC 3340 and they found that 2 % of glucose was the optimum concentration for the maximum yield.

Dikin *et al.* (2007) reported that the quantity of lactose affected the production of antifungal substances of *B. cepacia* and *B. multivorans*. This could be due to the feedback effect on the host cells, which might enhance carbon metabolism and consequently lead to the fast accumulation of the carbon. *Burkholderia* isolates had different capabilities to produce anti-candidal substances in the same medium. The varied capability of each strain

to hydrolyze and utilise different carbon sources may depend on active enzymatic systems of each strain.

Nitrogen is one of the key requirements for the growth and synthesis of cell component and functional proteins, and greatly influences microbial growth. Nitrogenous compounds may influence the biosynthesis of antibiotics at the level of secondary metabolism, as substrates for antibiotic biosynthesis, or through modulation of biosynthesis and activity of the enzymes. Aharanowitz, (1980) reported that amino acids can serve as a carbon source in the absence of any other carbon source in a defined medium. Both inorganic and organic nitrogen were tested in this study, and they both exhibited a significant effect on the anti-candidal activity. In comparison with inorganic nitrogen sources, organic nitrogen (yeast extract, urea, peptone and CAS amino acids) gave relatively higher anti-candidal activity. This corroborates the findings of Vahidi *et al*., (2004) and Saurav and Kannabiran (2010) who reported that organic nitrogen improved the production of antifungal agents by actinomycetes. Yeast extract, CAS amino acids and ammonium phosphate were selected as a nitrogen sources for the optimized anti-candidal production medium that was developed.

The combination between yeast extract, CAS amino acids and ammonium phosphate improved anti-candidal activity above the level obtained these nitrogen sources alone or in other combinations that did not include all three. The stimulatory effect of complex nitrogen sources such as yeast extract, CAS amino acids and peptone, on production and activity of anti-candidal agents might be due to the presence of the amino acids, peptides, vitamins, numerous trace elements in these complex organic mixtures. Such supplementary

compounds might exhibit a stimulatory effect or act as inducers of enzymatic systems which are responsible for anti-candidal production and activity.

The pH value has a marked effect on the production of anti-candidal agents. The current study found that the maximum activity of anti-candidal agents by *Burkholderia* was achieved when the initial pH was adjusted to 6 in BSM medium. The finding was consistent with Chin-A-Woeng *et al*., (1998) who found that pyrrolnitrin increased below pH 6. Quan *et al*., (2009) and Li *et al*., (2007) reported that the production and activity of CF66I from *B*. *cepacia* was influenced by the level of pH and the best production and activity was at pH 6. Moreover, different studies reported that *Burkholderia* tend to be found at highest numbers in acidic soils. Therefore, it was not unexpected to see the optimal active anti-candidal metabolite production at acidic pHs (Dalmastri*, et al.*, 2003, Ramette*, et al.*, 2005 , Li*, et al.*, 2007).

Bacterial growth and production of effective metabolites were dependent on temperature and how temperature affects the activity of enzymes. The growth of *Burkholderia* and the synthesis of anti-candidal agents need a series of enzyme catalysed reactions which are affected by temperature. However, lower and higher temperatures can reduce the activity of the enzymes, reduce the growth rates and hence production rate of metabolites. The optimum temperature for maximum anti-candidal production and activity of tested *Burkholderia* lay in the range of 15 to 25 ºC. *B. vietnamiensis* JW13 lost the ability to produce active anti-candidal agents at 40°C; this could be because temperate temperatures are suitable for growth of such bacteria and consequently for the activity of enzymes responsible for anti-candidal biosynthesis and activity. This corroborates the findings of

previous studies in this field. Quan *et al*. (2009) reported that the most suitable temperature of the production of CF66I from *B*. *cepacia* was 25°C. At this temperature activity of CF66I increased rapidly and was greater than at other temperatures used.

It is well known that *Burkholderia* can grow and utilise a variety of organic compounds under different physical conditions through different metabolic pathways (Chain*, et al.*, 2006). However, it is very useful to identify the optimal medium, temperature and pH preferences of *Burkholderia* to produce active anti-candidal agents. The regulatory mechanism of the production of anti-candidal agents in *Burkholderia* is poorly understood. The interpretation of the results obtained herein was complicated because of the potential for overlapping affects with the different factors tested. In addition to gene activation /repression by the growth conditions, the growth substrates tested may also enter different metabolic pathways which lead to intermediate compounds needed to produce the antifungal metabolites.

The main purpose of this work was to select the best medium composition and conditions to improve the activity of anti-candidal agents of *B*. *ambifaria* AMMD. The media optimization studies revealed that different media compositions and culture conditions have significant impacts on the activity of anti-candidal agents, and the most interesting finding was that *Burkholderia* exhibited promising activity on the culture media BSM4.6, BSM5.6 and BSM0.6 and the common effective factor for all these media was the pH of 6.

5.5 Conclusions

- The production of anti-candidal agents from *B. ambifaria* AMMB, BCF and Kc311 and *B. vietnamiensis* JW13 were reported for the first time.
- Glycerol and succinate at lower concentrations were the carbon sources that resulted in largest activity of anti-candidal agents of *Burkholderia.*
- Hydrogen ion concentration (pH) was also one of the most important growth conditions. It influenced *Burkholderia* anti-candidal antibiotic production with pH 6.0 being optimal. pH also effected the growth of the basidiomycete with BSM-G at pH 6.0 being one of the best growth media for *B. adusta* BK1.
- Increased anti-candidal activity occurred with the addition of ammonium phosphate, CAS amino acids, ammonium chloride, peptone or yeast extract as a sole nitrogen source.
- Sodium nitrate totally suppressed the activity of anti-candidal agents of *Burkholderia*.
- Lower concentrations of phosphate (10 mM) increased the activity of anti-candidal agents more than higher concentrations.

CHAPTER SIX

ISOLATION AND IDENTIFICATION OF BACTERIA THAT PRODUCE ANTIFUNGAL COMPOUNDS

6.1 Introduction

A variety of microbes can be found growing in rhizosphere habitats. Soil-borne microorganisms interact with plant roots and soil constituents at the root–soil interface (Barea*, et al.*, 2005). There are several beneficial soil-borne microorganisms in the rhizosphere, which can improve soil quality and enhance crop production and protection. The release of root exudates and decaying plant material provide sources of nutrients for the soil microorganisms as growth substrates; the root surface also forms a structural platform on which root-associated microbiota develops (Werner, 1998). The *Burkholderia* are a large group of Gram-negative bacteria present in both natural and clinical environments. *B. cepacia complex* are opportunistic human pathogens responsible for lung infections in cystic fibrosis patients and various infections in immune-compromised patients (Mahenthiralingam & Vandamme, 2005). Moreover, they are present in the rhizosphere of many crop plants, particularly maize (Di Cello*, et al.*, 1997, Bevivino*, et al.*, 2005, Dalmastri*, et al.*, 2007), rice plants (Estrada*, et al.*, 2001), woodlands (Richardson*, et al.*, 2002) and agricultural and urban soils (Miller*, et al.*, 2002).

Previous work has shown that *B. cepacia* complex bacteria produce a wide range of antimicrobial substances (Chapter 3 and 4). A successful screening approach using a classical overlay assay to detect antimicrobial activity was developed (Chapter 2) and optimized in terms of substrate and physiological parameters (Chapter 5) to enhance secondary metabolite production. In addition, TLC-bioautography has been developed as a rapid and useful means to fractionate and purify *Burkholderia* antibiotic compounds (Chapters 4 and 5). Using these optimized approaches, a range of soil and rhizosphere

habitats were screened for the presence of antibiotic producing bacteria. A general hypothesis for this chapter was "all soil and rhizosphere bacterial isolates are capable of producing one or more antifungal agents." The specific aims of this chapter were:

- To isolate antifungal producing bacteria from different soil and rhizosphere environments, with a view to isolate antifungal *Burkholderia* species.
- Identify the antifungal producing bacteria using 16S rRNA gene sequence.
- Extract antifungal agents synthesised by these producing isolates.
- Study the presence and diversity of antifungal agents in antifungal producing bacteria using TLC-bioautography assay.
- To examine other non-*B. cepacia* complex such as *Burkholderia gladioli* as antifungal producing species.

6.2 Materials and Methods

6.2.1 Sample collection

Multiple environmental samples were examined as follows:

i. Soil samples were collected from mixed deciduous woodland in the Coed Beddick**,** Tintern**,** UK (coordinates 51.710690, -2.681351). A total of 10 soil samples were examined. Bacteria were cultivated from soil samples by adding of sieved soil to BSM-G medium supplemented with cycloheximide, and polymxin, and gently homogenised, and incubated horizontally at 30ºC for 24h, then 200 µl of each culture were transferred to a sterile micro-tube containing DMSO and stored until use.

ii. Maize rhizosphere and soil samples were collected from Hargrove Farm, Bridgend, Wales, UK (coordinates 51.515393,-3.646903), with permission from the land owner. A total of 14 soil samples were examined. To isolate bacteria from the maize rhizosphere, maize roots were aseptically removed, 200 µl of the root homogenate was added to 2 ml of BSM-G medium and then incubated at 30ºC for 24 h. After growth, 200 µl of each culture was transferred to a sterile micro-tube, DMSO was then added to 8%, and it was then stored at -80ºC until use.

iii. Bacteria associated with rhizosphere of tropical rainforest plants were obtained from multiple sites at the Danau Girang Field Centre, Sabah, Malaysia (coordinates 5.414000, 118.037317; all samples were obtained by E. Mahenthiralingam, Cardiff University). A total of 96, 1 cm root samples were aseptically cut from a range of plants growing on the rainforest floor and homogenised (using a disposable micro tube

pestle) in 1 ml of BSM-G (pH 5.5) containing 0.1 mg ml^{-1} of cycloheximide to prevent fungal growth. The samples were shipped to the UK immediately and the growth of bacteria allowed to proceed during this period (8 days). Upon arrival in Cardiff, DMSO was added to a final concentration of 8% v/v and the samples stored at -80°C prior to screening. Anti-candidal activity of these mixed rhizosphere cultures was tested using growth on BSM-G followed by overlaying with *C. albicans*; pure cultures of the anti-candidal bacteria within these mixtures were then obtained by plating to single colonies and re-screening the isolates (performed by Ewa Karpinska, Cardiff University).

iv. Four isolates of *B. gladioli* from cystic fibrosis (CF) patients were obtained from the Cardiff University Collection (Mahenthiralingam, *et al*., 2011) and included in this study since they had shown evidence of antifungal activity in previous work.

6.2.2 Antifungal activity of bacterial isolates

The soil, root and CF isolates were screened for antifungal phenotypes against *C. albicans* using an agar overlay assay (section 2.5.1), and against *B. adusta* using an agar antagonism assay (section 2.5.2). Active isolates which showed antifungal activity in the above assays were plated to obtain single colonies to enable purification of each antifungal producing microorganism. Single colonies were then retested for antagonistic activity using the overlay assay.

6.2.3 Identification of isolated bacteria

Antifungal producers were putatively identified to the species level using 16S rRNA gene sequence analysis (section 2.4). All pure isolates were stored at -80ºC until use. All of

isolated antifungal producing bacteria were subjected to qualitative analysis using TLC-bioautography assay (section 2.10).

6.3 Results

6.3.1 Isolation of antifungal producing bacteria

In total, 120 samples of rhizosphere soil and plant root samples were collected from different locations and screened for antifungal activity. This resulted in the isolation of 84 pure bacterial isolates, of which 16 had anti-candidal activity in an initial pre-screen using the overlay assay. These 16 environmental isolates were combined with 4 *B. gladioli* strains isolated from CF patients and subjected to detailed antimicrobial activity analysis.

All 20 isolates inhibited growth of *C. albicans*, with different inhibition zones ranging from 9 to 60 mm (Table 6.1). The maximum inhibition was caused by *B. gladioli* (Bcc1671 and Bcc1668) and *B. cepacia* Bcc1640 (60.0, 40.0 and 33.5 mm respectively) (Table 6.1). All four isolates of *B. gladioli* obtained from CF infection demonstrated both anti-candidal and anti-basidiomycete activity (Figure 6.1 and Table 6.1).

The effect of bacterial metabolites on *B. adusta* varied considerably. Three different classes of outcomes were observed between the bacteria and *B. adusta* (as described in chapter 3). Firstly, a full inhibition of *B. adusta* growth (Figure 3.4 A) was defined when there was no growth at all of *B. adusta*. Secondly, deadlock (Figure 3.4 B) was defined when the bacterial metabolites stopped the growth of *B. adusta* and finally *B. adusta* sometimes overgrowth the bacteria (Figure 3.4 C).

Table 6.1 Antifungal activity of active bacterial species isolated from different environments

No	Cardiff collection	Original name	Putative Identification	Location	Source	Antifungal activity										
						Agar overlay assay		TLC-Bioautography assay								
								Rf of active metabolites								
						CA	BK1	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
1	BCC1630	10TC1	*B. ambifaria*	Danau Girang Field Centre, Sabah, Malaysia	ENV	**29**	**+**	0.0	0.1	-	-	-	-	-	-	-	-
2	BCC1631	10TC2	*B. cenocepacia*		ENV	**11.5**	**-**	0.0	0.12	-	-	-	-	-	0.7	-	-
3	BCC1632	45TC1	*B. cenocepacia*		ENV	**15**	**+**	0.0	0.12	-	-	-	-	-	0.7	-	-
4	BCC1635	7PMX2	*Burkholderia sp.*		ENV	**8.5**	**-**	-	0.12	-	-	-	-	-	-	-	-
5	BCC1638	5PMX3	*B. ambifaria*		ENV	**51**	**-**	-	-	-	-	-	-	-	-	-	0.92
6	BCC1640	10PMX2	*B. cepacia*		ENV	**33.5**	**-**	-	-	-	-	-	-	-	0.7	-	-
7	BCC1641	10PMX3	*B. cenocepacia*		ENV	**9**	**+**	-	-	-	-	-	-	-	-	-	0.92
8	BCC1667	64952	*B. gladioli*	Cardiff UK	CF	**25**	**+**	0.0	-	0.28	-	-	0.56	-	-	-	-
9	BCC1668	5567	*B. gladioli*		CF	**40**	**++**	0.0	0.12	-	0.37	-	0.5	-	0.71	0.75	-
10	BCC1671	COL 1090	*B. gladioli*		CF	**60**	**+**	0.0	0.13	0.23	-	-	-	-	-	0.75	-
11	BCC1672	Manchester	*B. gladioli*	Manchester UK	CF	**30**	**+**	-	0.12	-	0.37	0.49	-	-	0.7, 0.76	-	0.99
12	ESH736	Chep.1.cord	*Bacillus* sp.*	Coed Beddick, Tintern U.K	ENV	**26**	**++**	0.0	0.12	-	-	-	-	-	0.74	-	-
13	ESH737	Chep.2.soil	*Bacillus altitudinis**		ENV	**22**	**-**	0.0	0.11	-	-	-	-	0.68	-	-	-
14	ESH738	S2.3	*Bacillus pumilus* *	Maize rhizosphere Bridgend, Wales, UK	ENV	**11**	**+**	-	-	-	-	-	-	-	-	-	-
15	ESH739	S2.5	*Paenibacillus polymyxa* *		ENV	**14**	**++**	-	0.10	-	-	-	-	-	-	0.8	0.98
16	ESH740	R3.2	*Bacillus licheniformis* *		ENV	**12**	**+**	-	0.10	-	-	-	-	-	-	-	-
17	ESH741	S4.4	*Bacillus altitudinis* *		ENV	**16**	**+**	-	-	-	-	-	-	-	-	-	-
18	ESH742	S4.7	*Bacillus safensis* *		ENV	**15**	**-**	0.0	-	-	-	-	-	-	-	-	-
19	ESH743	R6.6	*Bacillus subtilis* *		ENV	**11.5**	**-**	-	-	-	-	-	-	-	-	-	-
20	ESH744	R7.7	*Bacillus sonorensis* *		ENV	**12**	**+**	-	-	-	-	-	-	-	-	-	-

CA, *C. albicans;* BK1, *B. adusta;* CF, cystic fibrosis; ENV, environmental; * Nearest relative by 16S rRNA sequence analysis.

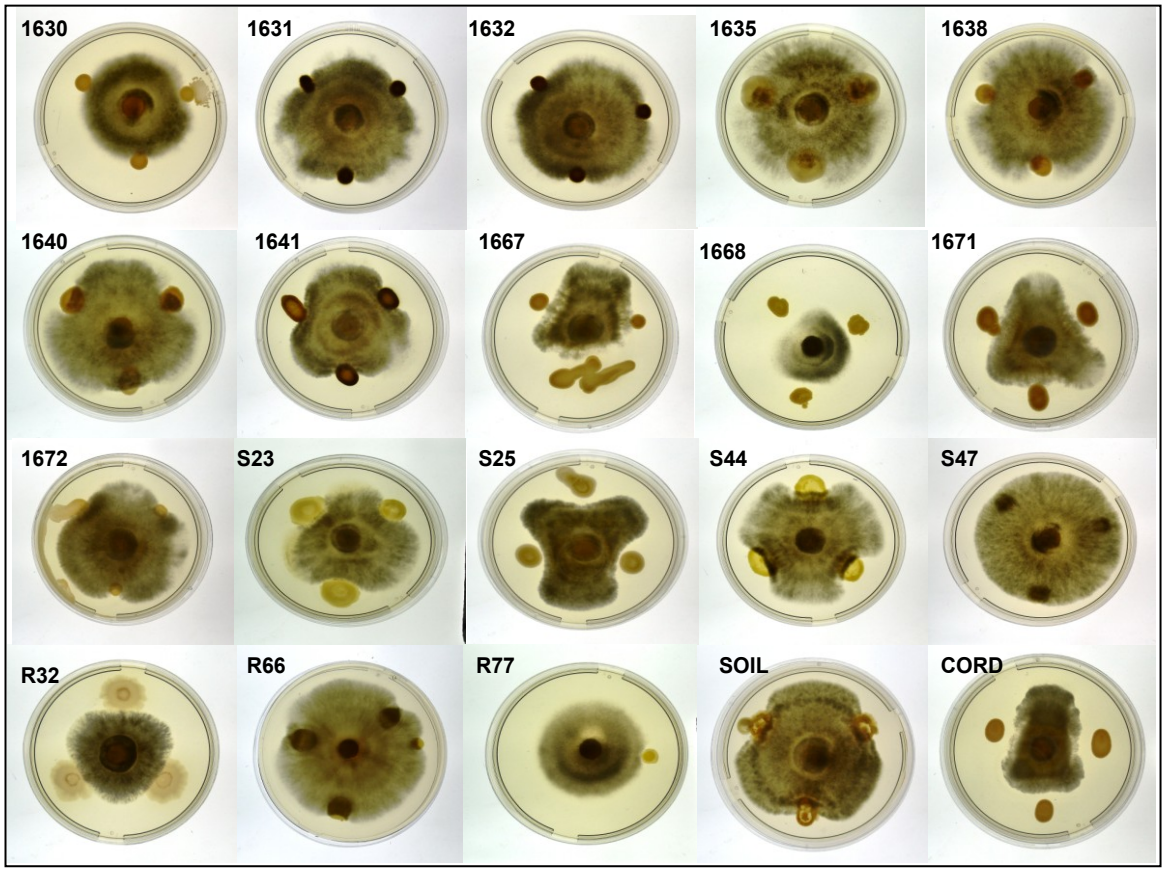


Figure 6.1 The outcomes (inhibition, deadlock and overgrowth) of the interaction between *B. adusta* BK1 and active bacterial isolates.

1630 (*B. ambifaria,* BCC1630), 1631 (*B. cenocepacia* Bcc1631), 1632 (*B. cenocepacia* Bcc1632), 1635 (*Burkholderia* sp. Bcc1635), 1638 (*B. ambifaria* Bcc1638), 1640 (*B. cepacia* Bcc1640), 1641 (*B. cenocepacia* Bcc1641), 1667 (*B. gladioli* Bcc1667), 1668 (*B. gladioli* Bcc1668), 1671 (*B. gladioli* Bcc1671), 1672 (*B. gladioli* Bcc1672), S23 (*Bacillus pumilus* ESH738), S25 (*Paenibacillus polymyxa* ESH739), R32 (*Bacillus licheniformis* ESH740), R44 (*Bacillus altitudinis* ESH741), S47 (*Bacillus safensis* ESH742), R66 (*Bacillus subtilis* ESH743), R77 (*Bacillus sonorensis* ESH744), SOIL (*Bacillus altitudinis* ESH737) , CORD (*Bacillus* sp ESH736).

These findings illustrate a remarkable diversity of interactions between bacteria and basidiomycetes. 65% of tested bacteria were able to produce and release active metabolites which inhibited the growth of *B. adusta*. The most aggressive *Burkholderia* isolate against *B. adusta* (Table 6.1 and Figure 6.1) was *B. gladioli* Bcc1668. Isolates of *B. gladioli* Bcc1667, isolate ESH739 and ESH736 produced very active antifungal agents which inhibited the growth of *B. adusta*. The weakest isolates were Bcc1630, Bcc1631, Bcc1632, Bcc1641, Bcc1671, Bcc1672, ESH739 and ESH740. Isolates Bcc1635, Bcc1638, Bcc1640, ESH742 and ESH743 were unsuccessful to inhibiting the growth of *B. adusta*. The results showed that many isolates were able to change the morphology of *B. adusta* growth, as there was clear variation in mycelial colour and hyphal density compared to the control. For example, Bcc1635, Bcc1640 and ESH737 isolates stimulated mycelia of *B. adusta* to produce fan-like structures at mycelial margins compared to the control, and other species were able to grow over *Burkholderia* colonies and cover it. *B. adusta* grew completely over ESH742 isolate (Figure 6.1).

The bacterial soil and rhizosphere isolates are listed in Table 6.1 with their classification based on the nearest match to their 16S rRNA genes. Seven antifungal isolates were obtained from the Malaysian rainforest rhizospheres, and six were members of the *Burkholderia cepacia* complex as follows: *B. ambifaria* (2 isolates), *B. cenocepacia* (3 isolates) and *B. cepacia* (1 isolate) (Table 6.1). The remaining rainforest isolate was a member of the genus *Burkholderia* but could not be identified to the species level based on 16S rRNA gene analysis (Bcc1635, Table 6.1). In contrast, no *Burkholderia* species were isolated from the maize rhizospheres in Bridgend, Wales, UK. *Bacillus* and *Paenibacillus* species isolates comprised the antifungal producers obtained. Four isolates were obtained

from loose soil around maize roots and included isolates putatively identified from their 16S rRNA sequences as *Bacillus* sp (ESH738, ESH739, ESH741 and ESH742). Three other isolates ESH740, ESH743 and ESH744 were recovered from the maize root-homogenates (as more tightly adhered rhizosphere bacteria) and were putatively identified as *Bacillus* sp. (Table 6.1). Two antifungal producer isolates, ESH736 and ESH737 (isolated from loose soil), were obtained from the forest rhizosphere of deciduous woodland in Tintern, UK and identified from their 16S rRNA gene sequences as *Bacillus* sp (Table 6.1).

6.3.2 Diversity of anti-candidal agents

Isolation of antifungal metabolites using resin extraction followed by TLC analysis was performed for all 20 anti-candidal isolates. The TLC-bioautography profiles demonstrated that 16 out of 20 isolates produced extractable metabolites which retained their anti-candidal activity (Table 6.1 and Figure 6.2). Resin crude extracts obtained from all 11 *Burkholderia* isolates and 5 *Bacillus* isolates showed anti-candidal activity. In contrast, the resin crude extracts of the remaining 4 *Bacillus* isolates (ESH738, ESH741, ESH743 and ESH744) failed to exhibit anti-candidal activity on TLC plates.

The *B. gladioli* isolates produced the greatest diversity of metabolites as well as the most potent antifungal compounds. *B. gladioli* Bcc1668 that produced a 40 mm inhibition zone against *C. albicans* (Table 6.1) had a TLC-bioautography with 6 active anti-candidal metabolites at different R*f*s: 0.0, 0.12, 0.37, 0.5, 0.71 and 0.75 (Figure 6.2). *B. gladioli* Bcc1672 produced a 30 mm inhibition zone and the TLC-bioautography showed 6 active metabolites at different R*f*s: 0.12, 0.37, 0.49, 0.70, 0.67 and 0.99.

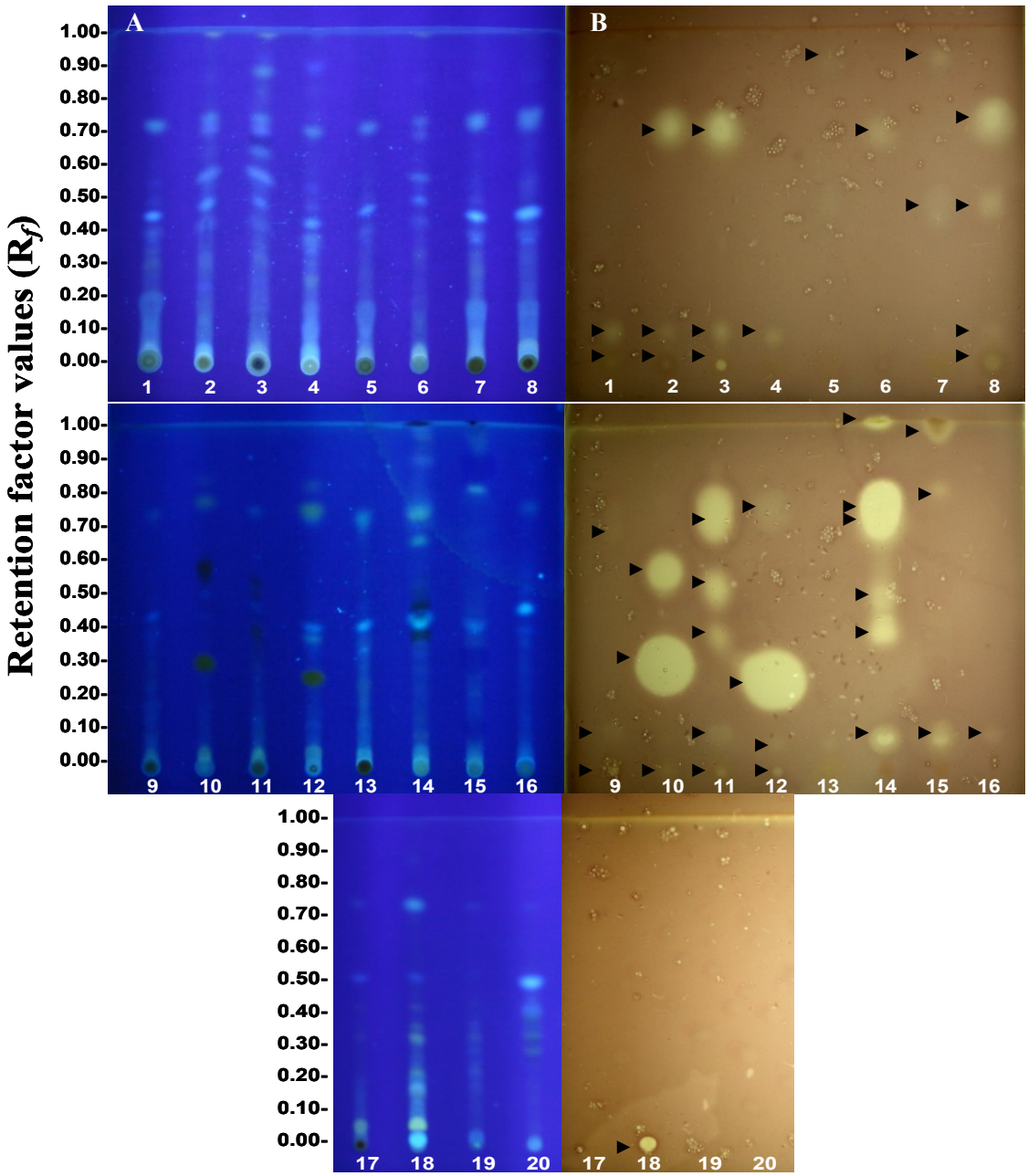


Figure 6.2 TLC-bioautography assay of anti-candidal metabolites secreted by bacteria isolated from different environments.

1 (*B. ambifaria,* BCC1630), 2 (*B. cenocepacia* Bcc1631), 3 (*B. cenocepacia* Bcc1632), 4 (*Burkholderia* sp. Bcc1635), 5 (*B. ambifaria* Bcc1638), 6 (*B. cepacia* Bcc1640), 7 (*B. cenocepacia* Bcc1641), 8 (*B. ambifaria* ESH736), 9 (*Bacillus altitudinis* ESH737) , 10 (*B. gladioli* Bcc1667), 11 (*B. gladioli* Bcc1668), 12 (*B. gladioli* Bcc1671), 13 (*Bacillus pumilus* ESH738), 14 (*B. gladioli* Bcc1672), 15 (*Paenibacillus polymyxa* ESH739), 16 (*Bacillus licheniformis* ESH740), 17 (*Bacillus altitudinis* ESH741), 18 (*Bacillus safensis* ESH742), 19 (*Bacillus subtilis* ESH743), 20(*Bacillus sonorensis* ESH744).

B. gladioli Bcc1671 produced a 60 mm inhibition zone and the TLC-bioautography showed 4 active metabolites at different R*f*s: 0.0, 0.13, 0.23 and 0.75. *B. ambifaria* Bcc1630 produced a 29 mm inhibition zone and the TLC-bioautography showed 2 active metabolites at R*f*s 0.0 and 0.10 (Table 6.1 and Figure 6.2).

Bacillus isolates showed different results using the TLC-Bioautography assay. ESH737 produced a 22 mm inhibition zone against *C. albicans* and the TLC-bioautography showed 3 active anti-candidal metabolites at different R*f*s: 0.0, 0.11 and 0.68 (Figure 6.2). ESH739 produced a 14 mm inhibition zone and the TLC-bioautography showed 3 active anti-candidal metabolites at R*f*s 0.1, 0.8 and 0.89 (Figure 6.2). The TLC- bioautography assay was not able to detect the active metabolites of the other 4 antifungal *Bacillus* isolates (Table 6.1 and Figure 6.1).

6.4 Discussion

Screening a variety of soil and rhizosphere isolates for antifungal activity revealed that *Bacillus* was the only genus of antifungal bacteria isolated from Bridgend UK maize fields. Other studies of microbial communities colonising maize rhizospheres all over the world have found that *Burkholderia* is one of the most common bacterial species present (Dalmastri, *et al.*, 1999, Estrada, *et al.*, 2002, Dalmastri, *et al.*, 2007). This unexpected finding might be due to the following: firstly, the main aim of this work was to isolate the antifungal producers. To achieve this, BSM-G medium was used because it had been found to enhance the antimicrobial activity whilst screening *Burkholderia* species (Chapter 3 and 5). The previous maize rhizosphere studies had used a semi-selective medium, *Pseudomonas cepacia* azelaic acid tryptamine (PCAT), which was formulated to enrich *Burkholderia* species. Another reason could be that the maize samples in this study were a different variety to the latter studies. Finally, the reason for the lack of *Burkholderia* could also be because maize was only introduced and cultivated just a few years ago in this area of the UK and the natural soils here may contain *Bacillus* as the dominant bacterial genus.

In general the attempts to extract antifungal agents from isolated bacteria using the resin method worked, but was unsuccessful in extraction the antifungal agents from 20% of isolates. The reasons for unsuccessful extraction might be due to the nature of the antifungal agents, to the solvent and the type of resin used in this study. Previous studies in this field corroborated, as they found that one of the basic parameters that influenced the extraction of active compounds was the solvent (Sukhdev, *et al.*, 2008). Different resins have different abilities in extracting antimicrobial agent(s) (Ribeiro & Ribeiro, 2003).

Different types of resin have been tested for extracting antimicrobial metabolites from *B. vietnamensis*, and it was found that the neutral resin of XAD-4 was able to extract anti-candidal metabolites when XAD-16 failed on an active isolate (Mahenthiralingam group, 2011 unpublished data). This could be due to the nature of different active metabolites, with the antifungal.

One out of 14 of rhizosphere isolates lost their antifungal activity after isolation as a pure culture. This could be because of the synergistic effects of certain metabolites which might activate such production. This was consistent with Cain *et al*., (2003) who found that mixture of rhizosphere bacteria became inactive after isolation of pure isolates .

6.5 Conclusions

- The most frequently isolated genera from Hargrove Farm, Bridgend, Wales, UK., maize rhizosphere and Coed Beddick**,** Tintern UK., deciduous woodland were *Bacillus* and *Paenibacillus*.
- Mixed microbial culture may lose their antifungal activity after isolation as a pure culture.
- The resin method was successful in extraction of the antifungal agents from *Bacillus* and *Burkholderia* species isolated from the rhizosphere.
- *B. gladioli* are an interesting source of antifungal metabolites. Crude resin extract comprised at least five main antimicrobial compounds.

CHAPTER SEVEN

GENETIC ANALYSIS OF ANTIMICROBIAL AGENTS PRODUCED BY *B. ambifaria* AMMD

7.1 Introduction

Burkholderia ambifaria AMMD (also known as Bcc0207; LMG 19182^T) was selected for genetic analysis of antimicrobial activity because this isolate has a fully sequenced genome. Currently, the complete genome of 20 strains of *Burkholderia* from different species and ecological categories has been sequenced. These *Burkholderia* sequences can be examined online at the *Burkholderia* Genomes Database (BGD; www.burkholderia.com). The *B. ambifaria* AMMD genome is very large (genome size of 7.57 Mb), has a high GC content (66.8%), is composed of 3 replicons, and contains a large number of insertion sequences. *B. ambifaria* AMMD was originally isolated from the pea rhizosphere and is the representative of *Burkholderia* species, with biocontrol potential against a range of microorganisms (Coenye*, et al.*, 2001). In the current study it was also shown that *B. ambifaria* AMMD has activity against Gram-positive and negative bacteria, *C. albicans* and range of basidiomycetes (Chapter 3).

Genomic information available on the publicly accessible databases such as Genbank or BGD can be investigated to increase our understanding of the genes responsible for biosynthesis of metabolites with antimicrobial activity. The expression of *B. ambifaria* AMMD genes induced during stationary phase growth on glycerol was recently mapped by microarray analysis (Mahenthiralingam*, et al.*, 2011). This growth condition is known to induce antimicrobial activity of strain AMMD (see Chapter 3 and 5) and therefore it was not surprising to see that 6 putative secondary metabolite gene clusters were significantly up-regulated. In the current study, using a TLC-bioautography assay, more than 6 secondary metabolites with antimicrobial activity were detected and identified (Chapter 5). Correlation of these active antimicrobial compounds to specific biosynthetic loci will considerably advance our knowledge of antibiotic production by *Burkholderia*.

One of the most powerful approaches used to identify the function of microbial-encoded genes is to introduce mutations into the bacterial genome and screen for mutants which have lost specific biological functions. Transposition is a recombination process in which transposable elements, such as Mu, Tn3, Tn5 or Tn10, move from one site on a genome to a new site on the same genome, or move from a mobile element such as plasmid into a completely new genome (Kleckner, 1981). The movement of transposons in this way can cause genetic rearrangements such as deletions, inversions or duplication of DNA sequences, as well as inactivation of the genes within which they insert and sometimes activation of genes adjacent to the site of insertion (Steiniger-White, *et al.*, 2004). Transposon mutants lacking the ability to produce certain antimicrobial agents have been used to demonstrate the role of antibiotics in the biocontrol functions mediated by microorganisms such as *Pseudomonas* (Fravel, 1988, Raaijmakers, *et al.*, 2002).

There are multiple bioinformatics tools available that can aid the identification of known secondary metabolite genes within genomes such as that of *B. ambifaria* AMMD. In addition such genome mining may also lead to the discovery of novel antibiotics within *Burkholderia* genomes. Artemis is a genome sequence visualisation and analysis tool developed by Rutherford *et al*. (2000) (http://www.sanger.ac.uk /resources/software/ artemis/). The software allows, on one screen, the viewing of the sequence of an entire genome in a graphical format; additional tools, such as the Artemis Comparison Tool (ACT), also enable the display of pair-wise comparisons between two or more genomes (Berriman & Rutherford, 2003). Genome annotation is the identification of putative functional roles for genes encoded within a DNA sequence (Lukashin & Borodovsky, 1998). The BGD (www.burkholderia.com) provides similar features to Artemis but allows

Burkholderia genomes to be viewed and compared over the web. Hence, this tool can allow the comparison of *B. ambifaria* AMMD to all the additional *Burkholderia* genomes available. It is a very powerful tool with which to identify the presence of orthologous genes in *Burkholderia* genomes.

A general hypothesis for this chapter was ‘‘transposon mutagenesis combined with genomic analysis can identify and predict the *B. ambifaria* AMMD genes involved in its antifungal activity.’’ The specific aims of this chapter were:

- To identify the gene(s) responsible for the production of anti-candidal agents by transposon mutagenesis of *B. ambifaria* AMMD.
- To characterise previously identified *B. ambifaria* AMMD mutants with altered anit-*B. multivorans* activity using resin-based metabolite extraction followed by TLC-bioautography assay.
- To bioinformatically analyse the glycerol-induced up-regulated *B. ambifaria* AMMD gene clusters observed by Mahenthiralingam, *et al*., (2011) and search for the presence of orthologs within other *Burkholderia* genomes.
- To use the knowledge from the above genetic analysis in combination with conventional phenotypic assays to characterise the kinetics *B. ambifaria* AMMD metabolites secretion.

7.2 Materials and Methods

7.2.1 Screening *B. ambifaria* AMMD transposon mutants

A bank of 2500 *B. ambifaria* AMMD mini-*Tn*5-Km2 transposon mutants that had been previously created (Mahenthiralingam*, et al.*, 2011) was screened for mutants that lacked anti-candidal activity (see section 2.8.1). Individual mutants with altered anti-candidal activity were re-plated from the master mutant bank and retested (twice) to confirm the stability of their anti-candidal negative phenotype; individual mutants were then stored at –80ºC for further characterization. In addition, a selection of 7 mutants with altered activity against the bacterium *B. multivorans* (Mahenthiralingam *et al*., 2011) were investigated using resin extraction of metabolites from 3 day plate cultures, followed by TLC-bioautography assay (section 2.10). The range of bioactive compounds secreted by these *B. ambifaria* mutants, in comparison to the wild type AMMD parent, was compared using both *C. albicans* and *B. multivorans* TLC overlay (see section 2.10).

7.2.2 Detection of *Tn*5 insertion site

To detect the transposon insertion site for each *B. ambifaria* AMMD mutant that had altered activity against *C. albicans*, *B. ambifaria* AMMD mutants were grown in TSB containing Kanamycin (Km; 50µg/ml) at 37°C for 24h. DNA was extracted from each mutant culture using a mechanical shearing, bead-beater based, extraction protocol (Mahenthiralingam *et al*., 1996). Random Amplified Polymorphic DNA (RAPD; Mahenthiralingam *et al*., 1996) PCR fingerprinting was performed, as described in section 2.8.2, to confirm that each transposon mutant was a derivative of *B. ambifaria* AMMD and not a contaminant isolated during the screening process. PCR was used to amplify DNA

adjacent to the transposon insertion sites and then this DNA was sequenced as described elsewhere (Manoil & Beckwith, 1985) section 2.8.3.

Briefly, the DNA adjacent to the *Tn*5 insertion was amplified using a two-step PCR reaction protocol (step PCR1 and step PCR2) and 4 different primers. PCR 1 amplified the end of the transposon and adjacent chromosomal DNA to give sequences of variable lengths, due to the random priming of primer 2b at genomic sites flanking the transposon (Figure 7.1). Products of PCR1 were further amplified using PCR2. PCR2 products were sequenced using primer PCR 3, on an ABI automated sequencer (Cardiff University Molecular Biology unit, DNA Sequencing Core). The amplified short sequences adjacent to the transposon insertions were matched to the *B. ambifaria* AMMD genome using BLASTN similarity search available at the BGD.

7.2.3 The Time course of production of anti-candidal agents

Changes in bacterial growth, culture pH and anti-candidal agents of *B. ambifaria* AMMD over time were studied using BSM-G broth medium at pH 6. Multiple 250 ml flasks containing 100 ml of BSM-G medium and inoculated with approximately 1 x 10^8 cfu of *B. ambifaria* AMMD were set up. The flasks were incubated either statically or with shaking at 30ºC. The initial pH of the growth medium before autoclaving was adjusted to pH 6. However, it was noted to move to pH 5.92 after autoclaving. During growth, the pH of the AMMD culture was not controlled by extensive buffering, with just a minimal phosphate buffer system used in the BSM growth medium. At specific time points, the bacterial growth as measured by turbidity at 630 nm was determined (the mean of 3 replicate reading was determined). At given time points the antimicrobial compounds were extracted

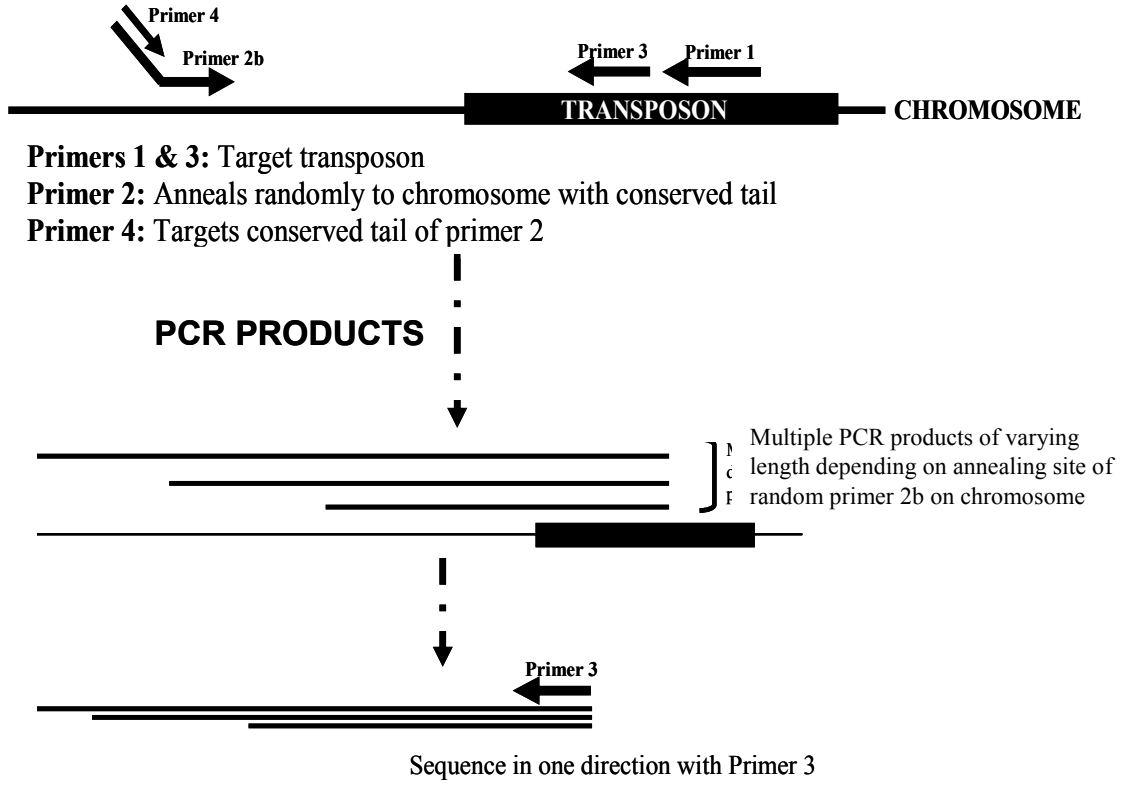


Figure 7.1 PCR Schematic for sequencing transposon insertion site.

This PCR determines the site of the transposon insertion in the bacterial genome. Chromosomal DNA adjacent to the Tn5 insertion is amplified using a two-step PCR reaction procedure shown in the diagram above. Primer Sequences: Primer 1, 5'-ttt tta cac tga tga atg ttc cg -3'; Primer 2b, 5'-ggc cac gcg tcg act agt acn nnn nnn nnn acg cc -3'; Primer 3, 5' – cgg att aca gcc gga tcc ccg -3'; Primer 4, 5' – ggc cac gcg tcg act agt ac.

from one set of cultures using XAD16 resin as described in section 2.9.2. The bioactive metabolites were then separated using TLC and located with a *C. albicans* overlay (section 2.10).

7.2.4 Bioinformatics analysis

The whole genome sequence of *B. ambifaria* AMMD was obtained from the *Burkholderia* Genome Database (http://www.burkholderia.com/). Artemis was used to further visualise and annotate the *B. ambifaria* AMMD genome. Information on *Burkholderia* antimicrobial agents was collected from previous literature and the current study. This information consisted of the name, chemical structure, nucleotide sequence of the biosynthetic gene(s) or the amino acid sequence(s) of the encoded proteins. The biosynthetic genes for the following known *Burkholderia* antibiotics were examined: pyrrolnitrin (Schmidt, *et al.*, 2009), quinolinone (Vial, *et al.*, 2008), enacyloxin (Mahenthiralingam *et al*., 2011) and the Afc-lipopeptide (Kang, *et al.*, 1998). The genes or proteins were searched against the AMMD genome using the BLASTN or BLASTP software at BGD. Located gene clusters were then marked onto the *B. ambifaria* AMMD genome using the Artemis software and Navigator tool to match up amino acid or nucleic acid sequence data. The GC content of the DNA was also visualised using the Artemis software. After location and further annotation, screen shots of the Artemis or BGD plots of the AMMD antibiotic loci were made to allow their presentation as figures.

7.3 Results

7.3.1 TLC-bioautography analysis of *B. ambifaria* AMMD mutants lacking activity against *B. multivorans*

Two of the AMMD mutants, 5.2.7 and 9.7.2, were negative for activity against both C. *albicans* and *B. multivorans* (Figures 7.2 and 7.3). Three of the AMMD mutants, 9.11.5, 10.11.3 and 21.7.2, showed negative anti-*B. multivorans* activity (Figure 7.3) but retained their anti-candidal phenotype in the overlay assay (Figure 7.2). Mutant 5.3.7 had reduced anti-*B. multvorans* activity (Figure 7.3) and normal anti-candidal activity (Figure 7.2). Mutant 11.8.2, a mutant which demonstrated both early appearance and enhancement of its anti-*B. multivorans* activity (Mahenthiralingam *et al*. 2011; Figure 7.3), possessed normal activity against *C. albicans* (Figure 7.2).

The genetic basis for each transposon mutation and the observed antimicrobial phenotype of each mutant is summarised in Table 7.1. Mutants 5.7.2 and 9.7.2 had the same negative phenotype against both *C. albicans* and *B. multivorans*; sequence analysis of their Tn*5* insertions demonstrated that they were both CepI mutants, the autoinducer synthase involved in quorum sensing by *B. ambifaria*. The resin metabolite extracts from both CepI mutants contained no anti-candidal (Figure 7.2) or anti-*B. multivorans* metabolites, demonstrating that all these antibiotics are dependent on an active quorum sensing system for their biosynthesis.

Mutants 9.11.5, 10.11.3 and 21.7.2 were only able to produce one anti-candidal compound detectable by TLC-bioautography (R*f* = 0.73; Figure 7.2) and completely lacked enacyloxin (R*f* = 0.5; Figure 7.3), the polyketide compound enacyloxin active against

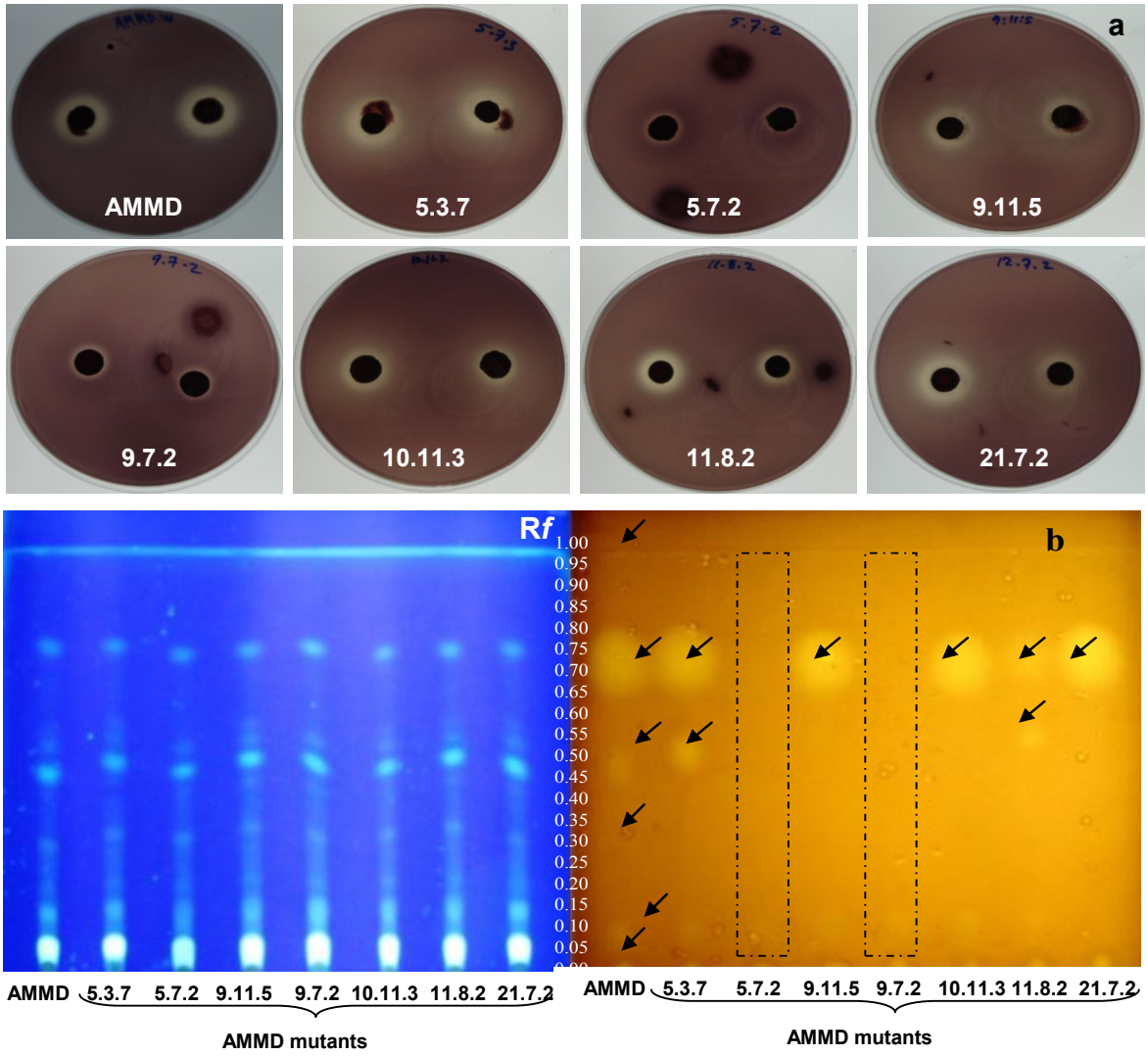


Figure 7.2 Analysis of anti-candidal activity of *B. ambifaria* AMMD mutants unable to inhibit *C. albicans*.

Overlay assay (panel a) demonstrated anti-candidal activity. TLC-bioautography assay (panel b) confirmed the presences and absences of anti-candidal agents in secondary metabolites of *B. ambifaria* AMMD wild type and 7 AMMD mutants. Arrows indicated anti-candidal activity.

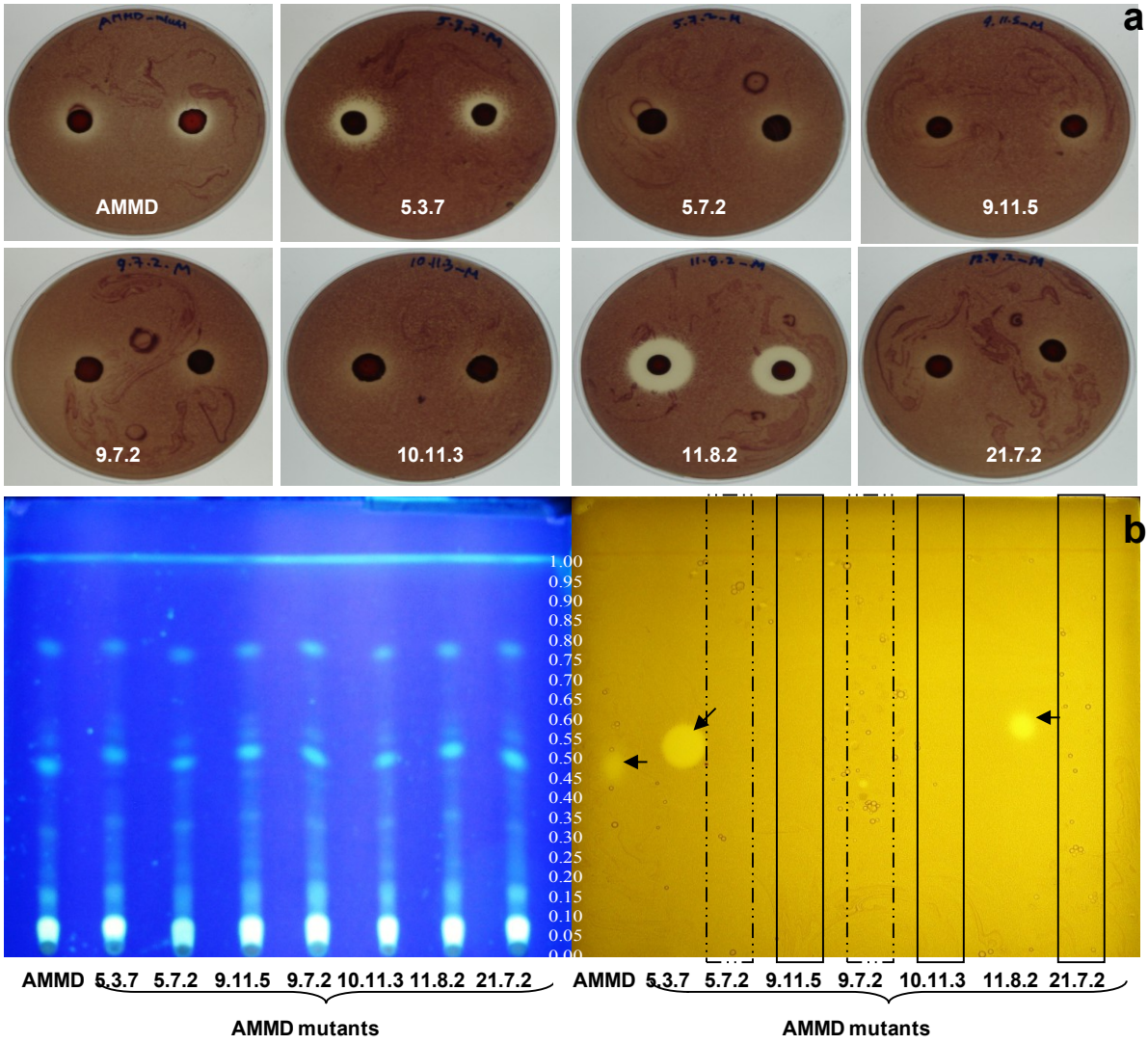


Figure 7.3 Analysis of anti-*B. multivorans* activity of *B. ambifaria* AMMD mutants unable to inhibit *B. multivorans*.

Overlay assay (panel a) demonstrated anti- *B. multivorans* activity. TLC-bioautography assay (panel b) confirmed the presences and absences of anti- *B. multivorans* agents in secondary metabolites of *B. ambifaria* AMMD wild type and other 7 AMMD mutants. Arrows indicated anti- *B. multivorans* activity.

B. multivorans (Mahenthiralingam, *et al*., 2011). Mutants 9.11.5 and 21.7.2 both contained transposon insertions in polyketide synthase (PKS) encoding genes within the enacyloxin biosynthetic cluster (Mahenthiralingam, *et al*., 2011); mutant 10.11.3 contained an insertion in a gene of hypothetical function which was also linked to enacyloxin production (Mahenthiralingam, *et al*., 2011). The presence of the one anti-candidal compound of R*f* 0.73 in these mutants suggested they retained the capacity to produce the quinolinone antifungal compound which separated as this fraction (chapter 4).

Mutant 5.3.7 contained a transposon insertion in a glycosyltransferase encoding gene; TLC analysis demonstrated that 5.3.7 had a similar anti-candidal compound profile to *B. ambifaria* AMMD wild type (Figure 7.2), but produced a larger enacyloxin-like fraction (R*f* = 0.52; Figure 7.3) despite its slightly weaker anti-*B. multivorans* phenotype in standard overlay assays. Mutant 11.8.2 contained an insertion in a cation diffuser family transporter gene and produced the normal range of anti-candidal (Figure 7.2) and anti-*B. multivorans* (Figure 7.3) compounds by TLC bioassay.

7.3.2 TLC-bioautography and genetic analysis of *B. ambifaria* AMMD mutants lacking activity against *C. albicans*

The mutant bank created by Mahenthiralingam, *et al*., (2011) had been screened for alterations in anti-*B. multivorans* activity using an overlay with this test organism. To fully explore the antimicrobial phenotype of *B. ambifaria* AMMD, its antifungal activity was further investigated by screening the same transposon mutant bank for defects in the production of anti-candidal agents. Out of 2500 isolates the anti-candidal activity of individual AMMD mutants varied and many of them showed un-stable activity against *C. albicans*. On the first screen of the mutant bank, 23 mutants with negative or reduced

activity were identified and selected for further analysis. After each of these mutants were re-screened individually for anti-candidal activity, 17 mutants were stably altered in anti-candidal activity and 6 reverted to wild type activity. A total of 9 out of 17 stable mutants were successfully subjected to DNA sequence analysis of their transposon insertions (Table 7.1). All 9 mutant sequences matched an insertion point on the AMMD genome (Table 7.1).

The genetic basis for each transposon mutation and their observed antimicrobial phenotype of each mutant is summarised in Table 7.1. Mutant 11.12.6, which encoded for an amino-acid N-acetyltransferase enzyme, was putatively involved in polyketide biosynthesis. Mutant 11.10.8 contained transposon insertions in a gene of hypothetical function (Bamb6146) which was linked to AfcA locus (Bamb_6128-6153).

7.3.3 The time course of production of anti-candidal agents

To gain an insight into the kinetics and physiology of *B. ambifaria* AMMD metabolite secretion, the production of anti-candidal compounds, cell density and pH were monitored over 170 hours of growth in BSM-G liquid medium static culture (Figure 7.4). The pH increased slightly from 5.92 to 5.98 in the early stage of the fermentation up to 14 h, then decreased to 5.2 at 78 h; for the last 4 days of culture the pH then increased to 5.56 by the end of the 170 h of test period. The optical density of growth culture increased over time, reaching a maximum at approximately 98 h, but then decreased for the remaining culture period.

Table 7.1 Characteristics of *B. ambifaria* AMMD transposon mutants

AMMD and mutants No.	Anti-candidal Activity		Anti-*B. multivorans* activity		Characteristics of AMMD mutants		
	Overlay assay	TLC bioassay (Rf)	Overlay assay	TLC bioassay (Rf)	Location of transposatnts (Chromosome No)	Gene name	Putative function
AMMD (wild type)	+	(6) 0.0, 0.08, 0.33, 0.5, 0.72 & 0.98	+	(1) 0.47	-		
Transposon mutants with altered *B. multivorans* activity isolated by Mahenthiralingam et al. 2011							
5.3.7	+	(2) 0.5 & 0.73	+ (reduced activity)	(1) 0.5	1	Bamb_2222	Glycosyltransferase, group 1
5.7.2	-	-	-	-	2	Bamb_4118	Autoinducer synthase, CepI
9.7.2	-	-	-	-	2	Bamb_4118	Autoinducer synthase, CepI
9.11.5	+	(1) 0.73	-	-	3	Bamb_5919	trans-AT modular PKS
10.11.3	+	(1) 0.73	-	-	2	Bamb_5367	Hypothetical protein
11.8.2	+	(2) 0.5 & 0.73	+	(1) 0.53	2	Bamb_5056	Cation diffusion facilitator family transporter
21.7.2	+	(1) 0.73	-	-	2	Bamb_5422	cis-AT modular PKS
Transposon mutants with altered anti-candidal activity isolated in this study							
11.10.8	-	ND	ND	ND	3	Bamb_6146	Hypothetical gene
11.12.3	-	ND	ND	ND	3	Bamb_5771	Acetyl-CoA acetyltransferases
11.12.6	-	ND	ND	ND	1	Bamb_2193	Amino-acid N-acetyltransferase
13.1.4	-	ND	ND	ND	1	Bamb_2465	Major facilitator superfamily MFS_1
13.5.1	-	ND	ND	ND	2	Bamb_3978	Intergenic inserton betweem glucose-methanol-choline oxidoreductase and a cytochrome C, class I protein encoding gene
17.11.6	-	ND	ND	ND	1	Bamb_1389	GTP-binding protein Typ A
18.1.2	-	ND	ND	ND	2	Bamb_5342	Hypothetical protein
18.7.6	-	ND	ND	ND	2	Bamb_5047	Peptidase C26
10.10.8	-	ND	ND	ND	1	Bamb_1541	Ton B-dependent siderophore receptor

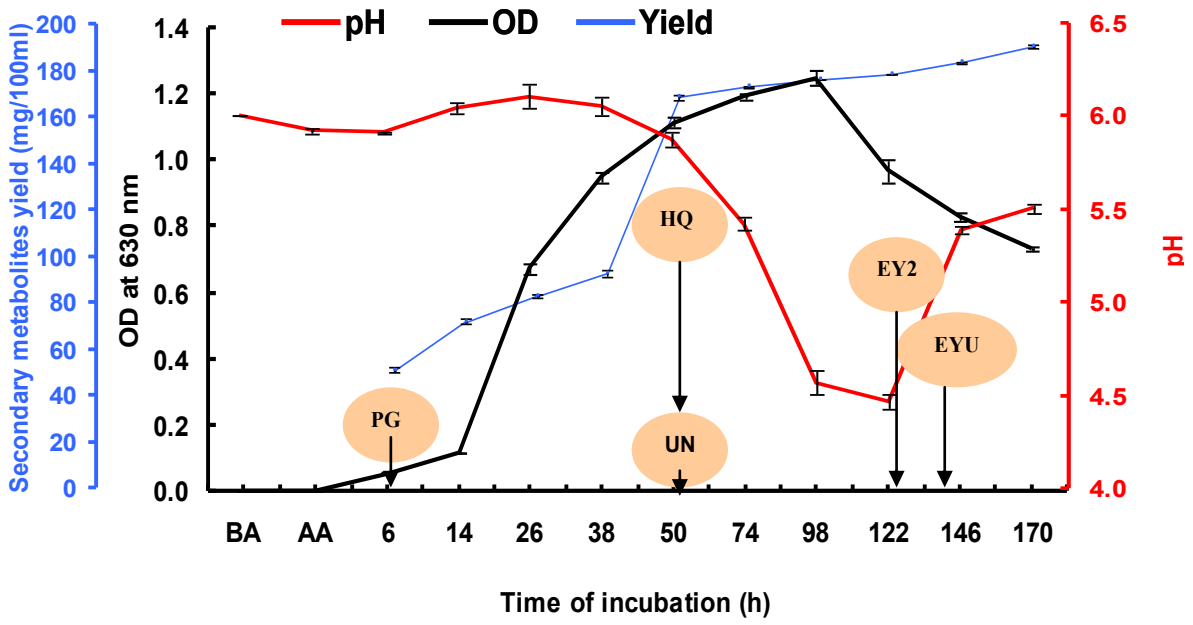


Figure 7.4 Time course of *B. ambifaria* AMMD culture tracking optical density, pH and secondary metabolites secretion.

B. ambifaria AMMD liquid cultures (100 ml) were grown statically for different lengths of time. The pH and culture optical density were measured. Replicates were harvested after various time periods of incubation and subjected to extraction of metabolites as following: The growth medium was centrifuged then the supernatant was used to extract bacterial secondary metabolites using Amberlite XAD16 resin. The resin was eluted with methanol to extract the anti-candidal components. By correlating the active fractions seen in the TLC-bioautography to the MS data, the appearance of the following compounds are indicated on the time course: polyethylene glycol (PG), 4-hydroxy-3-methyl-2-alkylquinoline (HQ), enacyloxin II (EY2), an enacyloxin derivative (EYU) and unidentified compounds (UN). The mean (± SEM) is shown for each parameter measured.

The dry weight yield of resin extractable metabolites increased gradually up to 38 h (787 mg l^{-1}), and then after 62 h increased rapidly to a maximum yield of 1619 mg l^{-1} (Figure 7.4). Using the TLC-bioautography assay at least five active anti-candidal metabolites of the *B. ambifaria* AMMD were detected (Figure 7.5). The first active anti-candidal agents were produced 6 h after inoculation (R*f* 0.10; Figure 7.5 B); from correlation with the MS data these metabolites were probably polyethylene glycol (PEG) and unknown peptides (see chapter 4).

After this, fractions corresponding to an unknown compound (R*f* 0.05) and quinoline (R*f* 0.72 ± 0.02) were detected after 50 h. A fraction corresponding to Enacyloxin II a (R*f* 0.50 ± 0.02) appeared after 122 h and another putative enacyloxin isomer (R*f* 0.35 ± 0.02) after 146 h (Figure 7.5). There was no sign of pyrrolnitrin production under these liquid growth conditions. Extraction and TLC-bioautography of the un-inoculated growth medium left under the same conditions did not demonstrate the presence of any anti-candidal metabolites, demonstrating that the activities observed were due to metabolites produced as a result of *B. ambifaria* growth (Figure 7.5).

7.3.4 Bioinformatics analysis of *B. ambifaria* genome

The TLC-Bioautography assay had detected more than six secondary metabolites with antimicrobial activity (chapter 4 and 5) in the *B. ambifaria* AMMD resin extracts from plate grown bacteria. The anti-candidal compounds were identified as pyrrolnitrin, quinolinone and enacyloxin using LC-MS (chapter 4). Bioinformatic characterization of known and novel antibiotic loci within the *B. ambifaria* AMMD genome was therefore

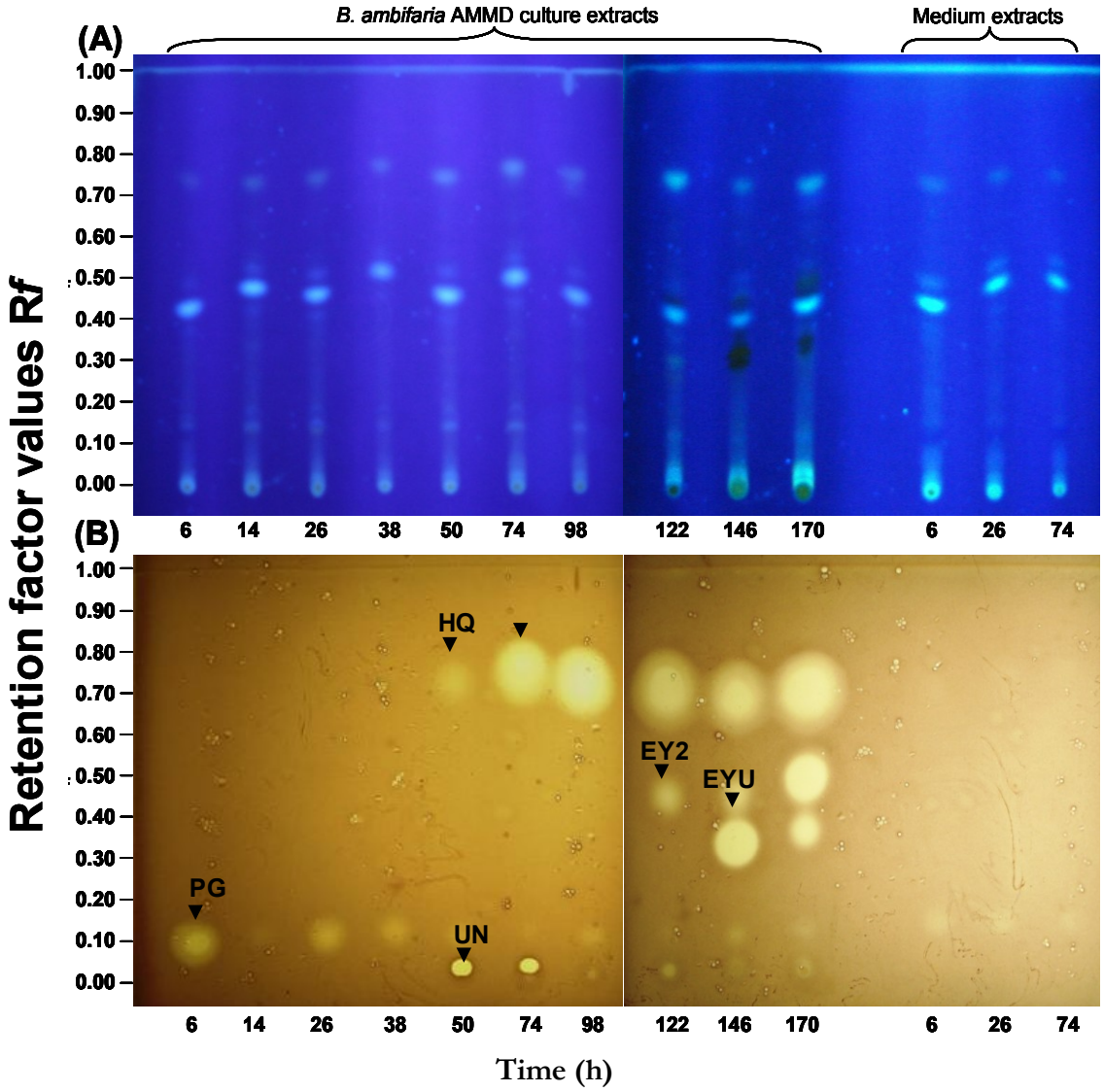


Figure 7.5 TLC-bioautography assay of anti-candidal metabolites secreted by *B. ambifaria* AMMD during time course.

B. ambifaria AMMD liquid cultures (100 ml) were grown statically for different lengths of time (h) as indicated at the bottom of each panel. Secreted metabolites were extracted, separated by TLC and visualized under UV light (Panel A). Extraction of growth medium without AMMD was performed as a control at three different time points (h) as indicated. The TLC plates were overlaid with *C. albicans* to reveal antifungal activity (Panel B). All TLC plates were run simultaneously as replicates and the retention factor (R*f*) scale is indicated. Arrows indicated the dot point of production of each active metabolite. The appearances of the following compounds are indicated on the time course: polyethylene glycol (PG), 4-hydroxy-3-methyl-2-alkylquinoline (HQ), enacyloxin II (EY2), an enacyloxin derivative (EYU), and unidentified compounds (UN).

performed to correlate the results of the chemical analysis with the putative functional biosynthetic capacity of the genome.

A pyrrolnitrin gene cluster was identified in *B. ambifaria* AMMD genome on chromosome 2 (Table 7.2). The pyrrolnitrin (*prn*) operon length was 5800 bases and contained four genes (Figure 7.6): *prnA* (Bamb_4726), *prnB* (Bamb_4727), *prnC* (Bamb_4728) and *prnD* (Bamb_4729). The GC content of the operon ranged from 56.9% to 73.4% (Figure 7.7), however, the average GC content was 65.7% and close to the average for AMMD genome (Table 7.2). Thirteen out of the twenty genome sequenced *Burkholderia* strains encoded the genes homologous to those in the *prn* operon. Seven strains were found to contain the whole pyrrolnitrin operon (genes *prnA* to *prnD*), while six strains contained an incomplete operon (Figure 7.8 and Table 7.3). For the seven strains with a complete *prn* locus, 5 encoded the operon on chromosome 2 and two encoded it on chromosome 3 (Table 7.3). Three of the genome sequenced Bcc strains had been previously tested for antifungal activity, *B. ambifaria* MC-40, *B. cenocepacia* MC0-03 and *B. lata* 383 and all were active (see chapter 3).

A 4-hydroxyl-3-methyl-2-alkyl-quinoline (HMAQ) gene cluster was identified within the *B. ambifaria* AMMD genome on chromosome 3 (Table 7.2). The length of HMAQ gene cluster was 11777 bases and contained 7 genes (Bamb_5763-5769; Figure 7.9). Although the GC content of the gene cluster varied from 55.2% to 76.3 %, the average GC content across the region was still typical for strain AMMD (Table 7.2). The Bamb_5767 gene within the cluster encoded a LuxR family transcriptional regulator (Appendix C; Figure C.1) indicating that the cluster was most likely regulated by quorum sensing. The absence

Table 7.2 Characterisation of *B. ambifaria* AMMD genes responsible for synthesis of antimicrobial agents

Chromosome No.	Cluster name	Characteristics					
		Number of genes	Gene name	Cluster length (Kb)	Average GC content (%; AMMD average = 66.7%)	No. putative orthologs	LuxR gene
3	Quinolines	7	Bamb_5763- Bamb_5769	11777	66.4	5	-
	Enacyloxin	33	Bamb_5910- Bamb_5943	84284	71.5	9	+
	AfcA lipopeptide	26	Bamb_6128- Bamb_6153	24238	70.6	13	-
	Unknown B	15	Bamb_6468- Bamb_6482	55082	67.1	5	+
2	Pyrrolnitrin	4	Bamb_4726- Bamb_4729	5800	65.7	13	-
	Unknown A	21	Bamb_3597- Bamb_3617	23444	67	4	-
1	No genes responsible for synthesis of antimicrobial agents detected						

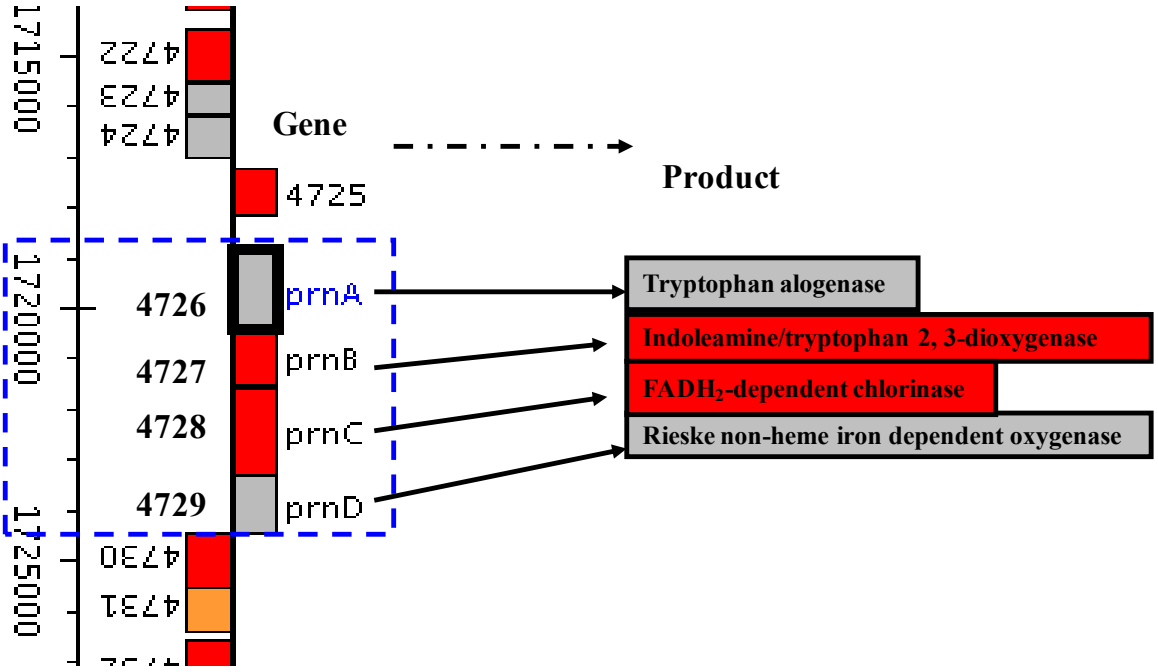


Figure 7.6 The pyrrolnitrin operon of *B. ambifaria* AMMD.

The amino acid and nucleotide sequences of pyrrolnitrin were searched against the *B. ambifaria* AMMD genome using the BLASTN or BLASTP software. The located gene cluster was then marked onto the *B. ambifaria* AMMD genome (Bamb_4726 to Bamb_4729) and navigator tool to match up amino acid or nucleic acid sequence data. Pyrrolnitrin operon genes (*prnA*, *prnB*, *prnC* and *prnD*), the functional annotation and products of genes are also shown. The colour indicated the subcellular localization classes as shown in the key.

Key:

Subcellular Localization Classes
Cytoplasm
Cytoplasmic membrane
Periplasmic
Outer Membrane
Extracellular
Unknown Localization
Unknown Localization (This protein may have multiple localization sites)
No localization assigned and computational localization prediction using PSORTB not performed

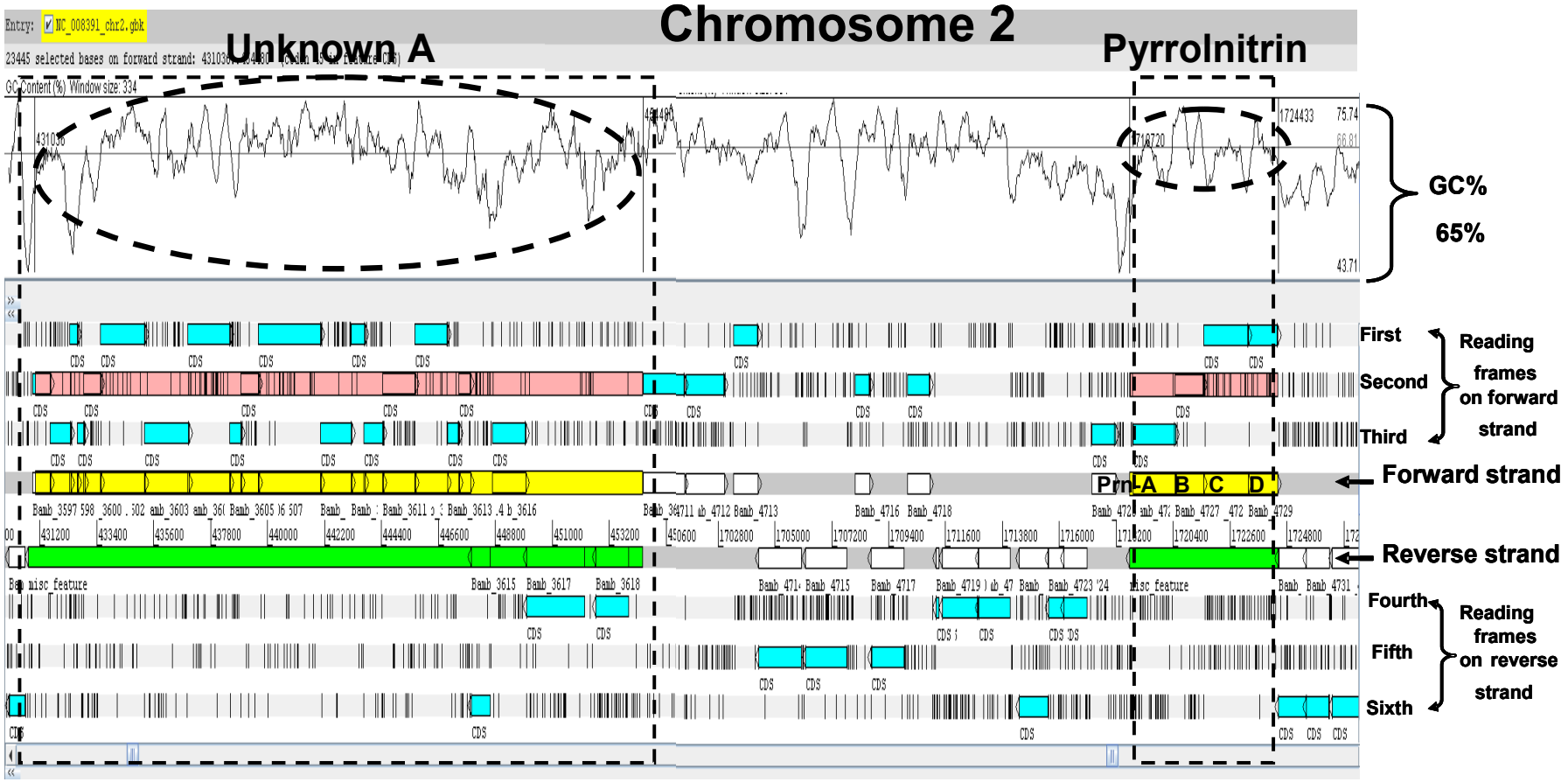


Figure 7.7 Antimicrobial biosynthesis loci encoded on chromosome 2 of *B. ambifaria* AMMD.

The linear representations of *B. ambifaria* AMMD chromosome 2 are viewed with Artemis software. The localisation of putative antimicrobial biosynthetic genes, pyrrolnitrin and unknown A, and their GC content are shown. The scale of the map is shown in 1 kb increments. The potential protein coding regions (colour-coded by biological role) are depicted as boxes with arrow heads indicating the direction of transcription.

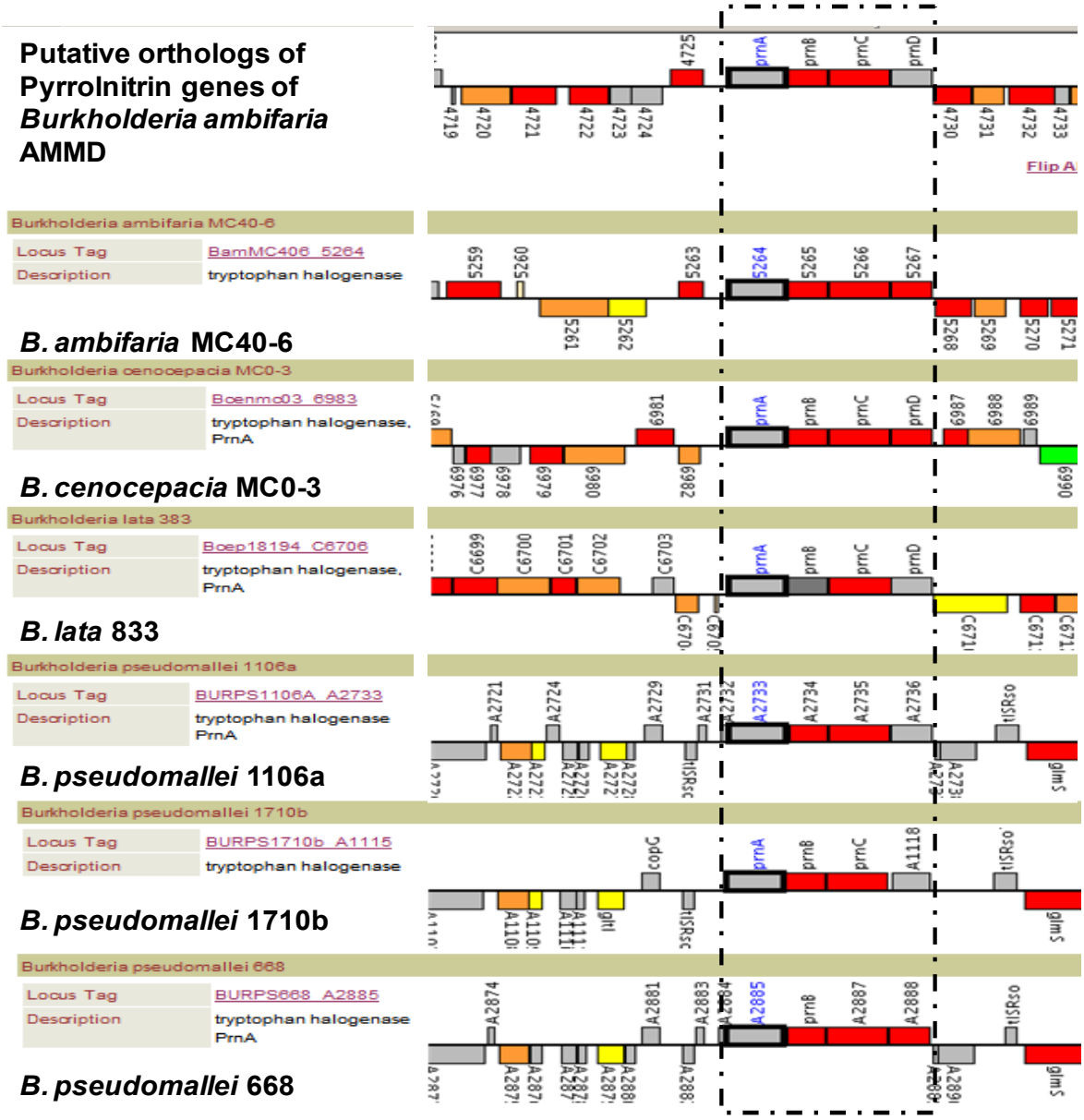


Figure 7.8 Orthologous arrangement of pyrrolnitrin biosynthesis genes of *Burkholderia* genomes.

Pyrrolnitrin genes were identified in 20 *Burkholderia* genomes. The top panel showed *B. ambifaria* AMMD pyrrolnitrin biosynthesis genes, and other six orthologous loci are shown. The colour indicated the subcellular localization classes as shown in the key.

Key:

Subcellular Localization Classes
Cytoplasm
Cytoplasmic membrane
Periplasmic
Outer Membrane
Extracellular
Unknown Localization
Unknown Localization (This protein may have multiple localization sites)
No localization assigned and computational localization prediction using PSORTB not performed

Table 7.3 Putative orthologs of *B. ambifaria* AMMD pyrrolnitrin genes in other *Burkholderia* genomes

Burkholderia strains	Pyrrolnitrin cluster				Chromosome number
	prnA (7 isolates ±)	*prnB* (7 isolates ±)	*prnC* (12 isolates ±)	*prnD* (13 isolates ±)	
***B. ambifaria* AMMD**	+	+	+	+	2
***B. ambifaria* MC40**	+	+	+	+	2
***B. cenocepacia* MC0-03**	+	+	+	+	3
***B. lata* 383**	+	+	+	+	3
***B. pseudomallei* 1106a**	+	+	+	+	2
***B. pseudomallei* 1710b**	+	+	+	+	2
***B. pseudomallei* 668**	+	+	+	+	2
***B. mallei* ATCC 23344**	-	-	+	+	2
***B. mallei* NCTC 10229**	-	-	+	+	2
***B. mallei* NCTC 10247**	-	-	+	+	2
***B. mallei* SAVP1**	-	-	+	+	2
***B. pseudomallei* K96243**	-	-	+	+	2
***B. cenocepacia* J2315**	-	-	-	+	2

of the quinolinone antibiotics in the metabolite extracts from the two CepI autoinducer synthase mutants corroborates this observation. A complete HMAQ gene cluster was found in three strains of fully sequenced *Burkholderia* strains (*B. ambifaria* MC40-6, *B. pseudomallei* K96243 and *B. thailandensis* E264; Appendix C; Figure C.2).

An enacyloxin gene cluster encoding 33 genes with a high GC content (71.5%) suggestive of its acquisition by horizontal gene transfer was identified on chromosome 3 (Figure 7.9 and Table 7.2). As with the HMAQ gene cluster, the enacyloxin cluster also encoded two LuxR family transcriptional regulator genes, Bamb_5910 and Bamb_5911 (Appendix C; Figure C.3), indicating regulation via quorum sensing and correlating with an absence of the polyketide in the two CepI autoinducer synthase mutants. Nine out of twenty genome sequenced *Burkholderia* strains possessed a limited number of genes orthologous to those within enacyloxin gene cluster (mainly single PKS-like genes), however, none had a completely identical cluster (Appendix C; Figure C.4). This also correlated with an absence of enacyloxin activity in the genome sequenced Bcc strains previously screened (chapter 3).

An AfcA lipopeptide gene cluster was also identified on chromosome 3. The gene cluster was 24238 bases in length and contained 25 genes: Bamb_6128 - Bamb_6153 (Appendix C; Figure C.5). Its average GC content was atypical for *B. ambifaria* AMMD at 70.6%, again suggesting that it was acquired by horizontal gene transfer (Figure 7.9). A complete AfcA lipopeptide gene cluster was found in four strains of fully sequenced *Burkholderia* strains (*B. lata* 833, *B. cenocepacia* MCO-3, *B. cenocepacia* AU1045 and *B. cenocepacia* HI2424; Appendix C; Figure C.6).

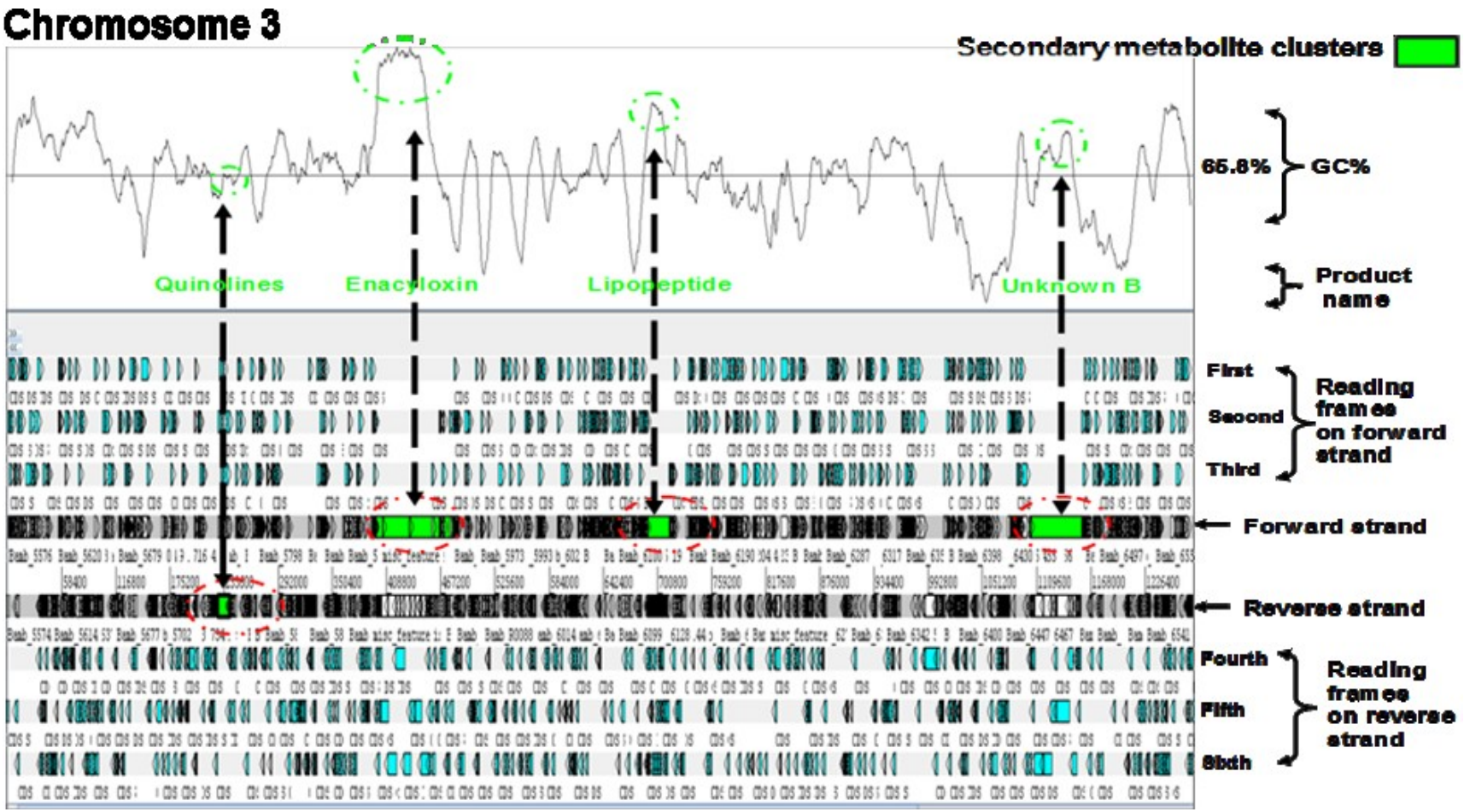


Figure 7.9 Antimicrobial biosynthesis loci encoded on chromosome 3 of *B. ambifaria* AMMD.

The linear representations of *B. ambifaria* AMMD chromosome 3 are viewed with Artemis software. The localisation of putative antimicrobial biosynthetic genes: 4 hydroxyl quinolines (Quinolines), enacyloxin, AfcA lipopeptide and unknown B, and their GC content are shown. The scale of the map is shown in 1 kb increments. The potential protein coding regions (colour-coded by biological role) are depicted as boxes with arrow heads indicating the direction of transcription.

Gene clusters for two unknown antimicrobials, A and B, were identified on *B. ambifaria* AMMD chromosome, 2 and 3, respectively (Table 7.2). The unknown A gene cluster length was 24238 bases with an average GC content of 67% (Figure 7.7 and Table 7.2). It contained 21 genes (Bamb_3597 to Bamb_3617) with Bamb_3608, Bamb_3611, and Bamb_3612 all predicted to encode polyketide syntheses hence suggesting the unknown A gene cluster encoded an antimicrobial metabolite. Only one genome sequenced *B. ambifaria* MC40-6, possessed a complete gene cluster homologous to that of the AMMD unknown A gene locus (Figure 7.7 and Appendix C; Figure C.9). The unknown B gene cluster was 55082 bases long and encoded 14 genes: Bamb_6468 - Bamb_6482; its GC content was typical of the rest of the AMMD genome at 67% (Figure 7.7 and Appendix C; Figure C.10).

7.4 Discussion

The purpose of this work was to identify the genes responsible for the production of the anti-candidal metabolites by *B. ambifaria* AMMD using transposon mutagenesis. The transposon insertions interfere directly or indirectly with gene function. However, it was difficult to explain the potential mode of action of all transposon insertion mutations. Six mutants lacked the ability to produce active antimicrobial agents when screened for the first time; these mutants reverted to wild type activity when individually re-screened for a second/third time. This instability of mutant phenotype against *C. albicans* may be due to the different Tn5 insertion sites in the AMMD genome, with some less stable than others. Another reason may have been that because BSM-G was used as a screening medium without the addition of kanamycin which is selective for the transposon used. A final reason for the transposon mutant instability observed could be the fact the *B. ambifaria* AMMD is known to secrete at least 6 anti-candidal agents (see chapters 4 and 5), and hence single mutations may not result in a clearly negative antifungal phenotype. This phenomenon was also reported by Cornelis, *et al*. (1992) who were working on stability, frequency and multiplicity of transposon insertions of fluorescent Pseudomonads, they found the transposons were unstable .

A TLC-bioautography assay was able to confirm the phenotype results of the overlay assay. The locations of active antimicrobial genes capable of inactivating antimicrobial production of 16 isolates of AMMD Tn5 mutants were detected. The insertions demonstrated that there were many genes involved in AMMD antimicrobial production and these were located on all chromosomes (1, 2 and 3) within its genome. Half of the

mutations altering antimicrobial production in the current study occurred in chromosome two, 31.25% were in chromosome one and 18.75% targeted chromosome three.

The TLC-bioautography assay results showed that Tn5 mutants 5.2.7 and 9.7.2 lacked all antimicrobial activity against both *C. albicans* and *B. multivorans*. Moreover, the results of the sequencing showed that the Tn5 insertion in both mutants occurred in the same site, a gene encoding the AMMD CepI homologue. CepI is responsible for the synthesis of QS molecules, N-octanoyl-homoserine lactone (HSL) derivatives which may have different carbon side chain lengths (C6 and C8-HSL have been observed in *B. ambifaria* AMMD; Vial et al., 2008). The lack of antimicrobial activity in the CepI mutants suggests that QS controls the biosynthesis of all antimicrobial agents of *B. ambifaria* AMMD. Different studies reported that many antimicrobial metabolites like bactobolin (Seyedsayamdost *et al.*, 2010), pyrrolnitrin (Keum*, et al.*, 2009, Schmidt*, et al.*, 2009) and the quinolines (Vial*, et al.*, 2008) are controlled by QS molecules.

The 5.3.7 and 11.8.2 mutants were able to produce two compounds (R*f* = 0.55±0.02 and 0.70±0.02) which were identified as enacyloxin and quinolines respectively (see section 4.42). Here, the Tn5 transposon was flanked in two different locations in the AMMD genome, Bamb_2222 and Bamb_5056 respectively, which were responsible for the synthesis of glycosyltransferase group 1 and a cation diffusion facilitator family transporter respectively. Glycosyltransferase group 1 encoded for an amino-acid N-acetyltransferase enzyme which is a matrix enzyme that catalyzes the synthesis of L-Glutamate to N-acetyl-L-glutamate in the presence of Acetyl-CoA. Bamb_2222 and Bamb_5056 were not a part of gene clusters responsible for the synthesis of enacyloxin and quinolines. These results

suggest that these genes might be indirectly involved in the production process by producing precursors or activators including such metabolites. In mutants 9.11.5 and 10.11.3 one compound with anti-candidal activity was produced (R*f* = 0.70 ± 0.02) and identified as quinoline (section 4.42). The Tn5 transposon insertion in 9.11.5 was in Bamb_5919, a gene encoding a trans-AT modular PKS that was part of polyketide biosynthesis pathway for the production of enacyloxin (Mahenthiralingam, *et al*., 2011).

During the time course of *B. ambifaria* AMMD fermentation, pH, bacterial growth and anti-candidal activity were monitored over time. The determination of the exact time of production of anti-candidal agents by *Burkholderia* under shaking conditions was difficult, because active anti-candidal compounds were produced and detected together at early time points such as 12 h of growth. The reason for this was not clear but it seems possible that these results were due to the early activation of the genes that were responsible for the production of such compounds. The very rapid rate of growth occurring with good aeration also meant that the cultures reached high cell density rapidly. It is well known that many secondary metabolites are produced during the latter stages of microbial growth. In addition the high cell density would mean that QS was active, which was to play an integral role in antimicrobial production from the isolation of the two CepI mutants.

Under static conditions it was possible to detect the time point of the production of several anti-candidal metabolites. Little variation in pH was recorded, and during *Burkholderia* growth the culture remained within the acidic pH range. As expected, the rate of anti-candidal metabolite production correlated with the growth rate of *B. ambifaria* AMMD and was the highest in the late stationary phase. The resin extract of the culture supernatant

inhibited *C. albicans* and other microorganisms, showing that the antimicrobial metabolites were extracellular. Lysis and extraction of the metabolites present within *Burkholderia* cells demonstrated that very little antimicrobial activity resided within the bacteria (data not shown).

Genomics provides a powerful tool to detect and predict genes responsible of antimicrobial production among a wide range of microorganisms. The results of a transcriptomic microarray study of *B. ambifaria* AMMD found that six gene clusters encoding putative bioactive metabolites were highly upregulated when the bacterium was grown on BSM-G (Mahenthiralingam, *et al*., 2011). These results correlate with the findings from the TLC-bioassays which separated at least 6 anti-candidal metabolites. Three of these metabolites were identified as enacyloxin, pyrrolnitrin and the quinolines (chapter 4), and upregulated gene clusters encoding the biosynthesis of these compounds were clearly located within the AMMD genome. The remaining 3 compounds, the AfcA lipopeptide and two unknown metabolites were not chemically identified in this study, but their gene clusters were characterized (Figure 7.10).

GC content varies in different organisms, and is one of the important parameters in helping understand the evolutionary relationships between bacteria species; closely related bacterial species have an average GC content which does not vary greatly between isolates of that species. Hence if GC content varies considerably within a region of genomic DNA, it is a hallmark of genes that may have come from a different bacterial species and is foreign to the encoding genome. The average *B. ambifaria* AMMD GC content was 66.8%. The GC content of pyrrolnitrin, unknown A and quinolines were within this

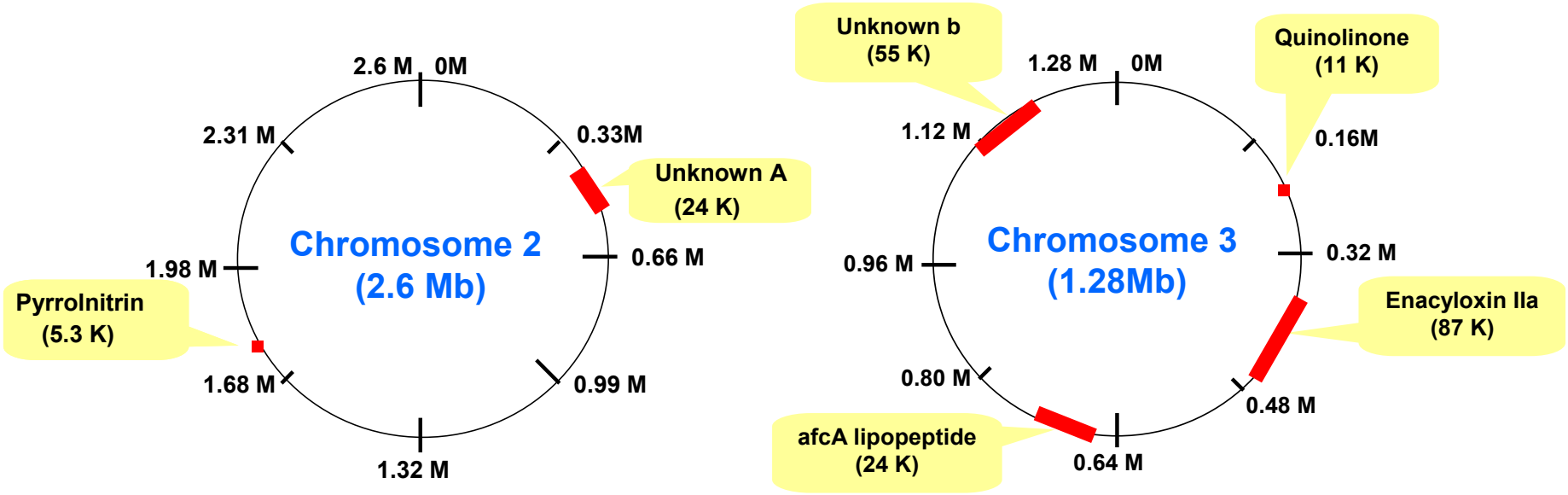


Figure 7.10 Distribution of the antimicrobial biosynthetic genes on *B. ambifaria* AMMD chromosome two and three.

The location and the size of the antimicrobial biosynthetic genes are shown.

average species range, suggesting that these antimicrobial biosynthesis genes may be quite ancestral in *Burkholderia* species such as *B. ambifaria.* In contrast, the GC content of enacyloxin, AfcA lipopeptide and unknown B antimicrobial biosynthesis clusters was above average for the AMMD genome, suggesting that they have originated in another species and been acquired by horizontal gene transfer.

Pyrrolnitrin is an antibiotic produced by a few genera of Gram negative bacteria, *Burkholderia* sp (Arima*, et al.*, 1964), Pseudomonads sp, Enterobacter sp and *Serratia* sp (Hammer*, et al.*, 1999). The *prn* operon was distributed among multiple genome sequenced *Burkholderia* species (Figure 7.8). In *B. ambifaria* AMMD the *prn* was composed of four genes with typical structure for pyrrolnitrin biosynthesis (Hammer, *et al*., 1999). The GC content and high frequency of the prn operon within other *Burkholderia* (65% of the available genomes) is indicative of the fact that the prn operon might have originated as a normal component of *Burkholderia* genomes. 6 out of 13 fully sequenced *Burkholderia* isolates had an incomplete set of the prn operon (*prnC* and *prnD*) and there was no information from the literature about the production of pyrrolnitrin in these isolates. These results suggest that: (i) that deletion or transfer of all or part the *prn* operon can occur, and (ii) an incomplete *prn* operon lacking the *prnC* and *prnD* genes is not sufficient to synthesise pyrrolnitrin. These findings are consistent with Costa *et al*. (2009) who found that *B. pseudomallei* K96243 missed a prn operon in comparison with the genome of other *B. pseudomallei* strains, and suggested that the *prn* operon was mobile. *B. ambifaria* AMMD genome contains several LuxR genes which are well-known controllers of quorum-sensing.

LC-MS detected and identified several quinolines derivatives in the *B. ambifaria* AMMD extracts and all showed anti-candidal activity (chapter 4). Through the time course of the experiment conducted with TLC-bioautography, quinolines were produced first at an early time of incubation of *B. ambifaria* AMMD. This finding suggests that quinolines play a basic role in anti-candidal activity in *B. ambifaria* AMMD. Further studies on these potent antimicrobial antibiotics, examining its inhibitory, toxicity affects and genetics are needed to see if they can be developed into a useful antibiotic.

7.5 Conclusion

The general hypothesis for this chapter was accepted in that transposon mutagenesis was found to be useful in locating genes which play a part in the biosynthesis of anti-candidal compounds by *B. ambifaria* AMMD. Additional conclusions are:

- The TLC-bioautography assay was able to confirm the phenotype results of the overlay assay for the AMMD transposon mutants.
- Sixteen mutants with altered antimicrobial phenotypes were identified from a total of 2500 transposon mutants screened. Eleven of the mutants were totally negative in anti-candidal activity, while the others had variable anti-candidal activity.
- The Tn5 insertions demonstrated that CepI mutants were totally defective in anti-candidal and anti-*B. multivorans* activity and demonstrated that QS plays an integral part in antibiotic production in *B. ambifaria* AMMD.
- The biosynthesis of antimicrobial agents: enacyloxin, AfcA lipopeptide and quinolines of *B. ambifaria* AMMD were controlled by QS molecules.
- Static growth conditions were best to screen for detection and the time point analysis of anti-candidal production by *B. ambifaria* AMMD.

- *B. ambifaria* encodes multiple genes which direct for antibiotic biosynthesis and these may be either ancestral to the species (such as those for pyrrolnitrin production) or have been acquired by horizontal gene transfer (such as those encoding enacyloxin biosynthesis).

CHAPTER EIGHT

CONCLUSIONS AND FUTURE WORK

Conclusions

The aim of the current work was to screen, isolate, characterise and identify *Burkholderia* antifungal agents, investigating their natural diversity and effect on both ascomycete and basidiomycete fungi. A comprehensive survey of the activity and diversity of antifungal agents present in a large collection of accurately identified *B. cepacia* complex and other *Burkholderia* was performed. The determination of the physiology and genetics of a small panel of antifungal *B. cepacia* complex isolates was also undertaken. The work provided detailed information on the range of *Burkholderia* antifungal metabolites and also developed new procedures to produce them and identify them.

8.1 Hypothesis one: All *B. cepacia* complex and other *Burkholderia* are capable of producing antifungal agents (Chapter 3).

The overall hypothesis was rejected, because not all *Burkholderia* species were found to be active in the secretion of antifungal agents.

To date, limited attention has been given to interactions between wood decay basidiomycetes and *Burkholderia.* In addition, no studies have looked at large collections of taxonomically well-defined Bcc isolates. Various media formulations were tested prior to the optimal media being selected to examine the diversity of anti-fungal activity across a collection of 397 *Burkholderia* isolates. An additional challenge of the work was that wood-decay basidiomycete fungi, which grow as mycelia, were used. Initial screening of anti-basidiomycete activity was performed using a conventional contact antagonism assay

that has been applied to other filamentous fungi. However, a novel overlay assay using homogenised mycelia to form an even growth of the basidiomycete was also developed. The work presented in this chapter focused on 339 Bcc and 58 other *Burkholderia* species. The antagonism towards 10 representative wood decay basidiomycetes and *C. albicans* was examined for the first time.

A total of 45% of tested *Burkholderia* isolates produced antifungal agents. The best media to support the production and the activity of anti-candidal agents by *Burkholderia* were SA and BSM-G. Growth on rich media like TSA did not result in antimicrobial activity. *B. ambifaria*, *B. cepacia* and *B. cenocepacia* were the most antagonistic Bcc species against both *C. albicans* and *B. adusta*. In contrast, *B. multivorans* and *B. stabilis* were highly limited in their ability to produce antifungal agents. A basidiomycete overlay assay was successfully developed for *B. adusta* BK1 and *T. versicolor* D2, and overall, *B. adusta* BK1 was selected as a model basidiomycete species to enable characterization of *Burkholderia*-fungal interactions.

8.2 Hypothesis two: *Burkholderia* produce more than one antimicrobial compound. TLC-bioautography can be used to examine *Burkholderia* antimicrobial diversity (Chapter 4).

The hypothesis was accepted because *Burkholderia* species isolates were found to be capable of producing more than one active antifungal agent, and TLC-bioautography was a good tool to study antimicrobial diversity.

Burkholderia are soil bacteria that are known to produce a range of potent antimicrobial agents, however, the extent of this activity across the genus and within the *B. cepacia* complex was not known. By screening for antifungal activity in the large collection of *Burkholderia* it was demonstrated that 45.5 % of isolates had antifungal activity (Chapter 3). Extraction and partial purification procedures were developed; extraction on Amberlite resin followed elution with methanol, applied for the first time to bind *Burkholderia* metabolites, proved to be an excellent means to obtain active antimicrobial extracts. TLC-bioautography was successfully applied to examine the diversity of *Burkholderia* antimicrobials in these extracts. The number of active metabolites observed using the TLC-bioautography ranged from 1 to six antifungal agents, with multiple Bcc strains capable of producing 2 or more antimicrobials. LC-MS was subsequently used to successfully identify *Burkholderia* antifungal metabolites.

Amberlite XAD-16 resin followed by methanol elution proved to be the simplest method for extracting the bioactive compounds at high purity from *Burkholderia*. TLC combined with a bioautography assay was a rapid and accurate method for the localization of antimicrobial activity on a chromatogram. It offered the ability to obtain a profile of *Burkholderia* antimicrobial secondary metabolite patterns to study the presence and diversity of antimicrobial agents of *Burkholderia*. The TLC-bioautography and LC-MS data showed that *Burkholderia* comprise a large reservoir of known and novel antimicrobial agents, especially for those exhibiting antifungal activity.

Several of the antifungal compounds discovered were described for the first time in this group of bacteria, with a striking example being the polyketide antibiotic, enacyloxin,

produced by *B. ambifaria* AMMD. *B. ambifaria* BCF produced 4 active compounds, with a similar bioautography profile to *B. ambifaria* AMMD, except for a highly active mixture of metabolites which were identified as bactobolin A, B, D and a new bactobolin containing chlorine. *B. ambifaria* KW and Mex-5 produced more than 6 active bands containing at least 10 detectable, but unknown, active antifungal compounds by LC-MS. Pyrrolnitrin, a well known *Burkholderia* and *Pseudomonas* antibiotic was detected in 60% of tested *Burkholderia* using comparative TLC with a pure pyrrolnitrin standard. Pyrrolnitrin was highly active on *C. albicans* and all basidiomycetes tested. In addition to the above, novel peptide antibiotics were detected in *B. ambifaria* AMMD, BCF, KW and Mex-5.

8.3 Hypothesis three: Chemical and physical factors can influence the anti-candidal production and activity of *Burkholderia* (Chapter 5).

This hypothesis was accepted with optimal carbon sources, pH, and phosphate concentrations defined for the production of anti-candidal agents by *B. ambifaria*.

The objective of this work was to improve the composition of BSM to enhance antimicrobial production by *Burkholderia.* Since BSM was a defined minimal salts medium it was possible to change the composition of its ingredients and its pH, and examine the effects of target nutrients on antimicrobial production**.** The production of anti-candidal agents of *B. ambifaria* AMMD was affected by both biotic and abiotic factors. Glycerol and succinate at low concentrations were the most optimal carbon sources for

antifungal activity. The pH was also an important growth condition, with pH 6.0 being optimal for the secretion of antimicrobials. Lower concentrations of phosphate (10mM or below) also improved the production of anti-candidal agents more than higher concentrations. Finally, sodium nitrate totally suppressed the activity of anti-candidal agents of *Burkholderia.*

8.4 Hypothesis four: All rhizosphere bacterial isolates are capable of producing one or more antifungal agents (Chapter 6).

The overall hypothesis was rejected, because not all isolated rhizosphere bacteria were found to be active in the secretion of antifungal agents.

The components of synthetic media and different factors such as pH, temperature and incubation period altered antibiotic biosynthesis in the model *Burkholderia* strains. The aim of this work was to use this knowledge of antibiotic production conditions to isolate and examine the natural diversity of antifungal producing bacteria associated with different rhizosphere environments. Enrichment with selective media and bacterial identification using the 16S rRNA gene were used to identify the isolated bacteria.

The most common antibiotic-producing genera isolated from the maize rhizosphere and deciduous woodland sample in this study were *Bacillus* and *Paenibacillus*. One rhizosphere bacterial mixture lost its antifungal activity after isolation as a pure culture. The resin method was also successful in extracting the antifungal agents from the active *Bacillus* isolates. However, in contrast to the hypothesis, not all the culturable rhizosphere

bacteria produced antifungal agents. In particular, the absence of cultivatable *Burkholderia* isolates from the maize rhizosphere samples was very different to that observed in other studies of this crop plant.

8.5 Hypothesis five: Can mutants and bioinformatics tools predict *B. ambifaria* AMMD antimicrobial activity and genes (Chapter 7).

The general hypothesis was accepted, with a combination of transposon mutation and bioinformatic analysis proving very useful in the characterization of genes involved in the antifungal activity of *Burkholderia.*

The main goal of this chapter was to identify the genetic basis for secretion of antifungal agents by *Burkholderia* and also characterize genes involved in antibiotic production from the available *Burkholderia* genome sequences. Transposon mutagenesis was used to identify genes involved in the anti-candidal activity of *B. ambifaria* AMMD. The transposon insertions demonstrated that there were multiple genes in the AMMD genome which controlled antimicrobial production. Of the mutants characterized, 50% occurred in chromosome two, 31.25% located to chromosome 1 and 18.75% located to chromosome 3.

The detection of the time point of the production of anti-candidal metabolites of *B. ambifaria* AMMD under static conditions was essential. Semi-purified extracts of *B. ambifaria* AMMD (R*f* 0.60 ± 0.03) containing enacyloxin IIa produced an inhibitory affect at a low concentration (15.6 μg/ml) against *E. coli, B. multivorans* and *A. baumannii.*

Bactobolin isolated from *B. ambifaria* BCF and Mex-5 showed a strong ability to inhibit *C. albicans* and other fungi belonging to basidiomycetes.

Bioinformatic analysis was a useful tool for characterizing antimicrobial biosynthesis genes that were upregulated in *B. ambifaria* AMMD under growth conditions optimal for antibiotic production. Several of these genes and gene clusters were highly conserved among other *Burkholderia* species. The average GC content of *B. ambifaria* AMMD genome was 66.7% and two of the gene clusters responsible for antimicrobial biosynthesis, the enacyloxin and AfcA lipopeptide clusters, were found to be higher in GC content suggesting they were originally from a foreign microorganism. The *B. ambifaria* AMMD genome contained several LuxR genes were potentially responsive to regulation by quorum-sensing molecules. Correlating to this finding, the production of enacyloxin, pyrrolnitrin, and the quinolines was not seen in *B. ambifaria* AMMD CepI AHL synthase mutant by TLC-bioautography analysis.

Future work

Based on the achievements of the current study a number of future research projects are suggested which include:

- Semi-purified extracts of *B. ambifaria* AMMD produced an inhibitory affect at a low concentration against *E. coli*, *B. multivorans* and *A. baumannii*. Further studies on this potent anti-Gram-negative antibiotic mixture are worthwhile, to further purify the mixtures of enacyloxins, quinolines and unknown antimicrobials present.

The inhibitory spectrum, toxicity affects and mode of action of these agents will need to be characterised if any of them can be developed into a useful antibiotic.

- Further purification and identification of novel antibiotics detected in *B. ambifaria* KW and Mex-5 is also worthwhile.
- One rhizosphere bacterial isolate mixture lost its antifungal activity after isolation into pure culture. The investigation of this phenomenon could be useful.
- Using bioinformatics it was possible to observe that multiple genome sequenced *Burkholderia* strains encoded genes for antimicrobial biosynthesis. These promising findings should be investigated using the optimal growth conditions and chemistry developed in this study to express and characterize the antibiotics encoded.
- The isolation, characterisation and identification of the *Burkholderia* antifungal metabolites linked to the inhibition of wood decay basidiomycetes should be undertaken, to explain their specific mode of action.
- Determination of whether there is a synergistic or antagonistic affect between the multiple antimicrobial metabolites produced by single *Burkholderia* isolates is worthwhile. This could also be expanded to see if mixing different *Burkholderia* isolates results in the expression of novel antimicrobial metabolites.
- Overall, this study has shown that just under half of all *Burkholderia cepacia* complex species isolates screened produced significant antifungal activity. This suggests they are potent sources of novel antimicrobials, with other species such as *Burkholderia gladioli* also being shown as capable of producing novel antibiotics. Future detailed characterization of these bioactive secondary metabolites should be carried out.

CHAPTER NINE

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APPENDICES

APPENDIX A

Table A. 1 The antifungal activity of the Mahenthiralingam lab bacterial collection.

No.	Esh's No.	Comments	Source	Identification	Anti-*Candida* activity (inhibition zone mm)			Anti-*Bjerkandera* (BK1) activity
					SA medium	BSM-G medium	LPA medium	SA medium
1	BCC1079	MDIII-T-209	ENV	*B. ambifaria*	**12**	**0**	**n**	**-**
2	BCC1089	MVP-C1-33	ENV	*B. ambifaria*	**0**	**0; 0**	**n**	**-**
3	BCC1088	MDIII-T-474(s)	ENV	*B. ambifaria*	**11**	**0**	**n**	**+**
4	BCC1087	MDIII-T-425(s)	ENV	*B. ambifaria*	**13**	**0**	**n**	**+**
5	BCC1086	MDIII-T-401(s)	ENV	*B. ambifaria*	**10**	**0**	**n**	**-**
6	BCC1085	MDIII-T-477	ENV	*B. ambifaria*	**5**	**0**	**n**	**+**
7	BCC1084	MDIII-T-281	ENV	*B. ambifaria*	**0**	**0**	**n**	**+**
8	BCC1083	MDIII-T-50	ENV	*B. ambifaria*	**0**	**0**	**n**	**+**
9	BCC1080	MDIII-T-2	ENV	*B. ambifaria*	**0**	**0**	**n**	**-**
10	BCC1078	MDIII-T-211	ENV	*B. ambifaria*	**24.6**	**8 (faint)**	**n**	**+**
11	BCC1077	MDIII-P-554(s)	ENV	*B. ambifaria*	**13**	**0**	**n**	**+**
12	BCC1076	MDIII-P-549(s)	ENV	*B. ambifaria*	**10**	**0**	**n**	**+**
13	BCC1075	MDIII-P-516(s)	ENV	*B. ambifaria*	**11**	**0**	**n**	**++**
14	BCC1074	MDIII-P-481(s)	ENV	*B. ambifaria*	**10**	**0**	**n**	**+**
15	BCC1073	MDIII-P-431	ENV	*B. ambifaria*	**10**	**0**	**n**	**+**
16	BCC1072	MDIII-P-170	ENV	*B. ambifaria*	**10**	**0**	**n**	**+**
17	BCC1070	MDIII-Bt-80	ENV	*B. ambifaria*	**10**	**0**	**n**	**+**
18	BCC1081	MDIII-T-8	ENV	*B. ambifaria*	**13**	**0**	**n**	**-**
19	BCC1098	MVP-C1-95	ENV	*B. ambifaria*	**0**	**34.5**	**n**	**+**
20	BCC1213	MC80-27	ENV	*B. ambifaria*	**0**	**0; 0**	**n**	**-**
21	BCC1212	MC40-6	ENV	*B. ambifaria*	**0**	**0; 0**	**n**	**++**
22	BCC0410	MVP/C1 64	ENV	*B. ambifaria*	**11**	**36**	**n**	**-**
23	BCC0411	MCI 13	ENV	*B. ambifaria*	**10**	**0**	**n**	**+++**
24	BCC1164	CEP1232	ENV	*B. ambifaria*	**5**	**12.5**	**n**	**+**
25	BCC1107	MVP-C2-79	ENV	*B. ambifaria*	**0**	**35.5**	**n**	**+**
26	BCC1105	MVP-C2-73	ENV	*B. ambifaria*	**0**	**0; 0**	**n**	**+**
27	BCC1103	MVP-C2-44	ENV	*B. ambifaria*	**0**	**34.5**	**n**	**++**
28	BCC1090	MVP-C1-40	ENV	*B. ambifaria*	**0**	**0**	**n**	**+**
29	BCC1099	MVP-C2-4	ENV	*B. ambifaria*	**5**	**10.5**	**n**	**+**
30	BCC1069	MDIII-Bt-62	ENV	*B. ambifaria*	**11**	**0**	**n**	**+**
31	BCC1096	MVP-C1-87	ENV	*B. ambifaria*	**7**	**0**	**n**	**+**
32	BCC1095	MVP-C1-80	ENV	*B. ambifaria*	**0**	**36**	**n**	**+**
33	BCC1093	MVP-C1-55	ENV	*B. ambifaria*	**6**	**38**	**n**	**+**
34	BCC1092	MVP-C1-53	ENV	*B. ambifaria*	**12 (faint)**	**0; 32**	**n**	**-**
35	BCC1216	MW20-13	ENV	*B. ambifaria*	**8 (faint)**	**0; 34**	**n**	**++**
36	BCC1064	MDIII-B-306	ENV	*B. ambifaria*	**0**	**0**	**n**	**+**
37	BCC1218	MW80-16	ENV	*B. ambifaria*	**0**	**53**	**n**	**-**
38	BCC1214	MA80-5	ENV	*B. ambifaria*	**19.6**	**17 (faint)**	**n**	**-**
39	BCC1100	MVP-C2-25	ENV	*B. ambifaria*	**0**	**37.5**	**n**	**+**
40	FC0623	HI 2345 (J82)	ENV	*B. ambifaria*	**18**	**26**	**n**	**+**
41	BCC1066	MDIII-B-399	ENV	*B. ambifaria*	**18.6**	**14.5 (faint)**	**n**	**+**
42	FC0767	R-0698	ENV	*B. ambifaria*	**0**	**0**	**n**	**+**
43	FC0766	R-0697	ENV	*B. ambifaria*	**0**	**0**	**n**	**-**
44	FC0669	BCF/HG1-A; LMG-P 24640	ENV	*B. ambifaria*	**0**	**39.5**	**n**	**-**
45	FC0662	ATCC 53266 LMG 17828;	ENV	*B. ambifaria*	**0**	**0**	**n**	**+**
46	FC0661	ATCC 51671	ENV	*B. ambifaria*	**0**	**0**	**n**	**+**
47	FC0646	BP023	ENV	*B. ambifaria*	**0**	**0**	**n**	**-**
48	BCC0481	HI-2433	ENV	*B. ambifaria*	**9.6**	**10**	**n**	**-**
49	FC0625	M54, HI 2347, R-5142	ENV	*B. ambifaria*	**18**	**25.5**	**n**	**++**
50	BCC0480	HI-2427	ENV	*B. ambifaria*	**0**	**20.5**	**n**	**++**
51	BCC0588	AMMD	ENV	*B. ambifaria*	**9**	**21.5**	**n**	**+**

52	BCC0589	AMMDR1	ENV	*B. ambifaria*	**6**	**20**	**n**	**+**
53	BCC0590	1324	ENV	*B. ambifaria*	**0**	**0**	**n**	**-**
54	CEP0996	LMG 19467	CF	*B. ambifaria*	**0**	**44**	**n**	**+**
55	CEP0958	LMG-P 24637	CF	*B. ambifaria*	**15**	**16.5**	**n**	**-**
56	CEP0617	LMG-P 24636	CF	*B. ambifaria*	**11**	**22**	**n**	**-**
57	CEP0102	ATCC 53267	ENV	*B. ambifaria*	**0**	**0**	**n**	**+**
58	BCC0399	R-10725	ENV	*B. ambifaria*	**11**	**29**	**n**	**+**
59	FC0627	Ral-3; R-8863	ENV	*B. ambifaria*	**0**	**33**	**n**	**-**
60	BCC1054	MCII-65	ENV	*B. ambifaria*	**14**	**0**	**n**	**+**
61	BCC1067	MDIII-B-625	ENV	*B. ambifaria*	**12**	**0**	**n**	**+**
62	BCC1222	MS40-6	ENV	*B. ambifaria*	**0**	**26 (faint)**	**n**	**-**
63	BCC1065	MDIII-B-388	ENV	*B. ambifaria*	**0**	**35**	**n**	**-**
64	BCC0477	AU0216	CF	*B. ambifaria*	**25.3**	**8 (faint)**	**n**	**++**
65	BCC1063	MDIII-B-289	ENV	*B. ambifaria*	**6**	**0**	**n**	**+++**
66	BCC1062	MDII-130riz	ENV	*B. ambifaria*	**14.3**	**0**	**n**	**++**
67	BCC1059	MDII-135pott	ENV	*B. ambifaria*	**6**	**0**	**n**	**+**
68	FC0768	LMG 19182; AMMD	ENV	*B. ambifaria*	**11.6**	**18**	**20.6 faint**	**++**
69	BCC1055	MCII-105	ENV	*B. ambifaria*	**14**	**0**	**n**	**+**
70	BCC1068	MDIII-Bt-42	ENV	*B. ambifaria*	**12**	**0**	**n**	**+**
71	BCC1053	MCII-16	ENV	*B. ambifaria*	**15**	**0**	**n**	**+**
72	BCC1052	MCII-68	ENV	*B. ambifaria*	**12**	**0**	**n**	**+**
73	BCC1051	MCII-17	ENV	*B. ambifaria*	**8**	**0**	**n**	**+**
74	BCC1050	MCII-4	ENV	*B. ambifaria*	**13**	**0**	**n**	**+**
75	BCC1049	MCI-7	ENV	*B. ambifaria*	**14**	**0**	**n**	**++**
76	BCC1041	MVP-C2-51	ENV	*B. ambifaria*	**8.6**	**35**	**n**	**-**
77	BCC0423	MCI 4	ENV	*B. ambifaria*	**0**	**12.5**	**n**	**++**
78	BCC0478	AU1366	CF	*B. ambifaria*	**13.3**	**33**	**n**	**-**
79	BCC1057	MCII-126	ENV	*B. ambifaria*	**10**	**0**	**n**	**+**
80	BCC1269	KC10-29	ENV	*B. ambifaria*	**11**	**0**	**n**	**+**
81	BCC0372	AU0212	CF	*B. ambifaria*	**0**	**0**	**n**	**+**
82	BCC1599	MEX-5	ENV	*B. ambifaria*	**0**	**33**	**n**	**-**
83	BCC1270	KC20-17	ENV	*B. ambifaria*	**0**	**23**	**n**	**+**
84	BCC1223	MS80-4	ENV	*B. ambifaria*	**0**	**21.75**	**n**	**-**
85	BCC1265	MC40-7	ENV	*B. ambifaria*	**0**	**15.5 (faint)**	**n**	**-**
86	BCC1259	KW20-2	ENV	*B. ambifaria*	**31.6**	**12.5 (faint)**	**n**	**-**
87	BCC1256	KW420-19	ENV	*B. ambifaria*	**31**	**48.5**	**n**	**-**
88	BCC1255	KW420-1	ENV	*B. ambifaria*	**0**	**10**	**n**	**+**
89	BCC1254	KW215-1	ENV	*B. ambifaria*	**10.3**	**13**	**n**	**-**
90	BCC1252	KW10-1	ENV	*B. ambifaria*	**30.75 (faint)**	**34**	**n**	**++**
91	BCC1248	LMG-P 24641	ENV	*B. ambifaria*	**8.3**	**13.5**	**n**	**-**
92	BCC1241	KC311-6	ENV	*B. ambifaria*	**23.6**	**44.5**	**15.6**	**-**
93	BCC1239	KC20-4	ENV	*B. ambifaria*	**7.5**	**16.5 (faint)**	**n**	**-**
94	BCC1232	KC0-18	ENV	*B. ambifaria*	**16**	**24 (faint)**	**n**	**+**
95	BCC1237	KC10-16	ENV	*B. ambifaria*	**0**	**30.5 (faint)**	**n**	**++**
96	BCC1228	KA20-1	ENV	*B. ambifaria*	**0**	**0**	**n**	**-**
97	BCC1236	KC5-54	ENV	*B. ambifaria*	**0**	**12.5 (faint)**	**n**	**++**
98	BCC1235	KC5-45	ENV	*B. ambifaria*	**0**	**37.5**	**n**	**++**
99	BCC1224	KS0-1	ENV	*B. ambifaria*	**0**	**0**	**n**	**-**
100	BCC1233	KC0-24	ENV	*B. ambifaria*	**0**	**24 (faint)**	**n**	**+**
101	BCC1048	MVP-C2-69	ENV	*B. ambifaria*	**8.6**	**28**	**n**	**+**
102	BCC0766	LMG 2129, ATCC 23061	ENV	*B. sandropogonis*	**0**	**0**	**n**	**-**
103	BCC1363	HI3849	ENV	*B. anthina*	**0**	**0**	**n**	**-**
104	BCC0639	LMG 20980	ENV	*B. anthina*	**0**	**0**	**n**	**-**
105	BCC0485	LMG 21821; AU1293	CF	*B. anthina*	**11.6 (faint)**	**0**	**n**	**-**
106	BCC0637	R-11761	ENV	*B. anthina*	**0**	**0**	**n**	**-**
107	BCC1351	AU5925	CF	*B. anthina*	**0**	**16.5 (faint)**	**n**	**-**
108	BCC1349	AU3904	CF	*B. anthina*	**0**	**0**	**n**	**-**

109	BCC0403	B11	ENV	*B. anthina*	**0**	**0**	**n**	**-**
110	BCC1358	AU6369	CF	*B. anthina*	**0**	**0**	**n**	**-**
111	BCC0635	LMG 16670t1	ENV	*B. anthina*	**0**	**12**	**n**	**-**
112	BCC0638	R-4190	ENV	*B. anthina*	**14.3 (faint)**	**17**	**n**	**-**
113	CEP0202	LMG 20983	CF	*B. anthina*	**12.6 (faint)**	**0**	**n**	**-**
114	BCC0636	LMG 16670	ENV	*B. anthina*	**0**	**11**	**n**	**-**
115	CEP0171	LMG 20982	ENVH	*B. anthina*	**21.25**	**16.5**	**n**	**-**
116	BCC1350	HI3541	ENV	*B. anthina*	**0**	**28.5**	**n**	**++**
117	CEP0193	J2543/GOVAN	ENV	*B. arboris*	**16.3**	**25 (faint)**	**n**	**+++**
118	FC0454	LMG 14939	CF	*B. arboris*	**13.3 (faint)**	**0**	**n**	**-**
119	BCC1306	28	ENVI	*B. arboris*	**0**	**0**	**n**	**++**
120	BCC0767	LMG 19076	ENV	*B.caledonica*	**0**	**0**	**n**	**-**
121	BCC0768	LMG 18531	ENV	*B. caribensis*	**10.3 (faint)**	**0**	**n**	**-**
122	BCC0769	LMG 2155, ATCC 25418	ENV	*B. caryophylli*	**12 (faint)**	**0**	**n**	**-**
123	BCC0753	IST475	CF	*B. cenocepacia*	**0**	**0**	**n**	**-**
124	BCC0749	IST462	CF	*B. cenocepacia*	**8.75 (faint)**	**12.5 (faint)**	**n**	**+**
125	CEP0107a	POPR8	ENV	*B. cenocepacia*	**20.3**	**30 (faint)**	**n**	**+++**
126	CEP0136	C1655/GOVAN; CEP0136	ENVH	*B. cenocepacia*	**8**	**30 (faint)**	**n**	**+++**
127	CEP0138	LMG 16659	CF	*B. cenocepacia*	**16**	**12**	**n**	**+++**
128	CEP0075	MCC136/VIDB6 566; CEP0075	NON	*B. cenocepacia*	**0**	**0**	**n**	**-**
129	BCC0999	MCII-168	ENV	*B. cenocepacia*	**22**	**27 (faint)**	**n**	**++**
130	BCC1004	MDI-11a	ENV	*B. cenocepacia*	**0**	**0**	**n**	**-**
131	CEP0158	J1054/GOVAN; CEP0158	NON	*B. cenocepacia*	**9.3**	**10 (faint)**	**n**	**+**
132	BCC0961	150-1238 (CZ1 isolate)	CF	*B. cenocepacia*	**10.6**	**7**	**n**	**+**
133	CEP0024	LMG 18829	CF	*B. cenocepacia*	**18.6**	**0**	**n**	**+++**
134	FC0127	SCRIPPS; FC0127	CGD	*B. cenocepacia*	**0**	**25 (faint)**	**n**	**+**
135	CEP0942	68 (pt X) H111CEP0942	CF	*B. cenocepacia*	**8.6**	**20 (faint)**	**n**	**+**
136	CEP0931	SG1	CGD	*B. cenocepacia*	**0**	**0**	**n**	**+**
137	FC0426	LMG 14271	CF	*B. cenocepacia*	**11**	**35**	**n**	**+**
138	FC0428	ATCC 13945	NON	*B. cenocepacia*	**12**	**14 (faint)**	**n**	**++**
139	FC0430	ATCC 25609	NON	*B. cenocepacia*	**15.5**	**25 (faint)**	**n**	**+**
140	FC0435	ATCC 25610	NON	*B. cenocepacia*	**11.6**	**17 (faint)**	**n**	**+**
141	CEP0791	C1631GOVAN	CF	*B. cenocepacia*	**0 (faint)**	**9 (faint)**	**n**	**++**
142	BCC1010	MDII-143pott	ENV	*B. cenocepacia*	**18**	**18.5 (faint)**	**n**	**+**
143	BCC0665	LMG 16656	CF	*B. cenocepacia*	**9.6**	**0**	**n**	**+**
144	BCC0687	IST439	CF	*B. cenocepacia*	**9.3**	**14.5 (faint)**	**n**	**+**
145	FC0504	K30-6	CF	*B. cenocepacia*	**18**	**10 (faint)**	**n**	**+++**
146	CEP0610	LMG 14274	CF	*B. cenocepacia*	**8**	**14 (faint)**	**n**	**-**
147	CEP0511	LMG 18830	CF	*B. cenocepacia*	**10**	**18 (faint)**	**n**	**+**
148	CEP0497	CEP0497	NON	*B. cenocepacia*	**0**	**0**	**n**	**++**
149	FC0663	LMG 18832; ATCC 17765	NON	*B. cenocepacia*	**9**	**22 (faint)**	**n**	**-**
150	FC0666	BC-1	ENV	*B. cenocepacia*	**10**	**25.5 (faint)**	**n**	**+++**
151	FC0667	BC-2	ENV	*B. cenocepacia*	**17.6**	**20.5 (faint)**	**n**	**+++**
152	CEP0244	J626/GOVAN; CEP0244	NON	*B. cenocepacia*	**0 (faint)**	**55 (faint)**	**n**	**-**
153	CEP0238	LMG 16654	CF	*B. cenocepacia*	**10.6**	**0**	**n**	**+**
154	FC0446	LMG 13011	CF	*B. cenocepacia*	**16.6**	**24.5 (faint)**	**n**	**+**
155	BCC0376	AU1097	CF	*B. cenocepacia*	**11.3**	**0**	**n**	**+++**
156	C6508	WG1; C6508	CF	*B. cenocepacia*	**0**	**0**	**n**	**-**

157	BCC1210	MC0-3	ENV	*B. cenocepacia*	**14.3**	**30**	**n**	**+**
158	C4455	DB1	CF	*B. cenocepacia*	**0 (faint)**	**10 (faint)**	**n**	**-**
159	C6965	MU1	CF	*B. cenocepacia*	**11.6**	**20 (faint)**	**n**	**+**
160	CEP0608	LC; CEP0608	CF	*B. cenocepacia*	**0 (faint)**	**0**	**n**	**-**
161	BCC1322	46	ENVI	*B. cenocepacia*	**9.3**	**28**	**n**	**-**
162	BCC1318	42	ENVI	*B. cenocepacia*	**21.3**	**30 (faint)**	**n**	**-**
163	BCC1014	MDII-151pott	ENV	*B. cenocepacia*	**12.3**	**n**	**n**	**-**
164	BCC1266	MC80-32	ENV	*B. cenocepacia*	**15.6**	**32**	**n**	**+**
165	C5424	LMG18827	CF	*B. cenocepacia*	**8**	**0**	**n**	**+**
166	BCC1203	HI2424	ENV	*B. cenocepacia*	**10**	**n**	**n**	**-**
167	BCC1295	15	ENVI	*B. cenocepacia*	**10.6**	**9 (faint)**	**n**	**+**
168	C6433	LMG18828	CF	*B. cenocepacia*	**0**	**0**	**n**	**+**
169	BCC1291	10	ENVI	*B. cenocepacia*	**16.6**	**24 (faint)**	**n**	**+**
170	C4414	RC1	CF	*B. cenocepacia*	**10**	**14 (faint)**	**n**	**+**
171	BCC1196	D1541	CF	*B. cenocepacia*	**13**	**9 (faint)**	**n**	**+**
172	C3921	MU1	CF	*B. cenocepacia*	**0**	**30 (faint)**	**n**	**+**
173	BCC0379	HI-2455	ENV	*B. cenocepacia*	**8.3**	**14**	**n**	**+++**
174	BCC1283	2	ENVI	*B. cenocepacia*	**12.5**	**16.5 (faint)**	**n**	**+**
175	BCC0451	MD II 429	ENV	*B. cenocepacia*	**22.6**	**24 (faint)**	**n**	**++**
176	BCC1028	MDIII-T-48	ENV	*B. cenocepacia*	**15.3**	**37**	**n**	**+**
177	BCC0460	CG 10/3	CF	*B. cenocepacia*	**12.3**	**17 (faint)**	**n**	**-**
178	BCC1143	C9330	CF	*B. cenocepacia*	**0**	**0**	**n**	**+**
179	C0131	GK1; C0131	CF	*B. cenocepacia*	**8**	**32 (faint)**	**n**	**++**
180	BCC1019	MDIII-B-716	ENV	*B. cenocepacia*	**18.3**	**23.5 (faint)**	**n**	**+**
181	BCC1016	MDII-129riz	ENV	*B. cenocepacia*	**11**	**18.5 (faint)**	**n**	**+**
182	BCC0382	FC0663	NON	*B. cenocepacia*	**9.6**	**18 (faint)**	**n**	**++**
183	BCC1211	MC5-8	ENV	*B. cenocepacia*	**16**	**24.5 (faint)**	**n**	**+**
184	BCC1305	27	ENVI	*B. cepacia*	**14.6 (faint)**	**11**	**n**	**-**
185	BCC1365	AU2837	CF	*B. cepacia*	**25**	**19.5**	**n**	**+**
186	BCC1404	60	ENVI	*B. cepacia*	**17**	**25.5 (faint)**	**n**	**+**
187	BCC0394	No. 2	NON	*B. cepacia*	**13.6**	**21**	**n**	**+**
188	CEP0509	LMG18821	CF	*B. cepacia*	**10.3**	**20**	**n**	**+**
189	CEP0615	DJF; CEP0615	CF	*B. cepacia*	**10.6**	**18 (faint)**	**n**	**+**
190	FC0660	ATCC 49709	ENV	*B. cepacia*	**17**	**9**	**n**	**++**
191	CEP0840	700; CEP0840	NON	*B. cepacia*	**10.7**	**8.5 (faint)**	**n**	**+**
192	CEP0877	MC 0353	ENV	*B. cepacia*	**13**	**13 (faint)**	**n**	**++**
193	BCC1275	AU 3206	CF	*B. cepacia*	**9.5**	**15 (faint)**	**n**	**-**
194	BCC1273	AU 6671	CF	*B. cepacia*	**19.6**	**21 (faint)**	**n**	**-**
195	BCC0660	LMG 6964	ENV	*B. cepacia*	**15**	**28 (faint)**	**n**	**++**
196	CEP0080	ATCC 17759; LMG 2161	ENV	*B. cepacia*	**19**	**12**	**n**	**++**
197	FC0461	LMG 17997	NON	*B. cepacia*	**0**	**0**	**n**	**-**
198	CEP0972	AVC1717	CF	*B. cepacia*	**21**	**12 (faint)**	**n**	**++**
199	BCC0464	VT	CF	*B. cepacia*	**0**	**10**	**n**	**+**
200	CEP0031	ATCC 25416; LMG1222	ENV	*B. cepacia*	**10.3**	**12**	**n**	**+**
201	BCC0977	MVPC2-7	ENV	*B. cepacia*	**19**	**13**	**n**	**++**
202	BCC0974	MDIII-B201	ENV	*B. cepacia*	**24**	**12.5 (faint)**	**n**	**++**
203	BCC0972	MDIII-P477	ENV	*B. cepacia*	**15.6**	**17 (faint)**	**n**	**++**
204	BCC0971	MDIII-T99	ENV	*B. cepacia*	**14.6**	**17 (faint)**	**n**	**++**
205	BCC0970	MCII-61	ENV	*B. cepacia*	**27**	**17 (faint)**	**n**	**++**
206	BCC0412	MVP/C1 77	ENV	*B. cepacia*	**20.3**	**9.5**	**n**	**++**
207	CEP0232	J1050/GOVAN	NON	*B. cepacia*	**10**	**16**	**n**	**++**
208	BCC0679	IST431	CF	*B. cepacia*	**9.6**	**19.5**	**n**	**+**
209	FC0668	BC-B	ENV	*B. cepacia*	**20 (faint)**	**22**	**n**	**+++**
210	C8509	MD; C8509	CF	*B. cepacia*	**12.3**	**18.5 (faint)**	**n**	**+**

211	BCC0367	AU0113	CF	*B. cepacia*	**28**	**10.5**	**n**	**+**
212	BCC1058	MCII-166	ENV	*B. cepacia*	**22.6**	**16.5**	**n**	**-**
213	CEP0533	WD1; CEP0533	CF	*B. cepacia*	**29**	**18.5 (faint)**	**n**	**++**
214	CEP1054	CEP1054	CF	*B. contaminans*	**24**	**23.5**	**n**	**++**
215	BCC1281	LMG 23254	ENV	*B. contaminans*	**0**	**18 (faint)**	**n**	**+++**
216	CEP0964	VP1; CEP0964	CF	*B. contaminans*	**20.5**	**15**	**n**	**+++**
217	BCC0649	IST410	CF	*B. contaminans*	**11.6**	**17 (faint)**	**n**	**++**
218	BCC0823	LMG 23255	CF	*B. contaminans*	**20.3**	**0**	**n**	**+++**
219	BCC1315	38	ENVI	*B. contaminans*	**18.6**	**12**	**n**	**++**
220	FC0431	ATCC 29352, R-2607	ENV	*B. diffusa*	**0**	**0**	**n**	**-**
221	CEP1010	LMG 18943	CF	*B. dolosa*	**0**	**0**	**n**	**+**
222	CEP1011	AU0746; R-6140	CF	*B. dolosa*	**0**	**22 (faint)**	**n**	**-**
223	FC0380	PC543	CF	*B. dolosa*	**0**	**27 (faint)**	**n**	**-**
224	CEP0766	AU0090, AU1293	CF	*B. dolosa*	**12.6**	**0**	**n**	**+**
225	BCC1359	AU3960	CF	*B. dolosa*	**0**	**23 (faint)**	**n**	**-**
226	BCC1343	AU794	CF	*B. dolosa*	**0**	**32 (faint)**	**n**	**+**
227	BCC1356	AU3556	CF	*B. dolosa*	**8**	**0**	**n**	**-**
228	BCC1361	AU2130	CF	*B. dolosa*	**9.3**	**0**	**n**	**+**
229	FC0377	PC195	CF	*B. fungorum*	**0**	**0**	**n**	**-**
230	CEP0847	no name	?	*B. fungorum*	**12 (faint)**	**0**	**n**	**-**
231	BCC0770	LMG 16225	CF	*B. fungorum*	**13.3 (faint)**	**0**	**n**	**-**
232	CEP0930	MA4	CF	*B. gladioli*	**32**	**70.6**	**n**	**+**
233	BCC1620	AU16905	CF	*B. gladioli*	**65**	**82**	**n**	**+**
234	BCC1621	AU17102	CF	*B. gladioli*	**70**	**70.6**	**n**	**+**
235	CEP0962	MC3	CF	*B. gladioli*	**10.3**	**41.3**	**n**	**-**
236	BCC0771	LMG 2216, ATCC 10248	ENV	*B. gladioli*	**12.3 (faint)**	**0**	**n**	**-**
237	BCC1624	AU17145	CF	*B. gladioli*	**20**	**31**	**n**	**-**
238	BCC1622	AU17110	CF	*B. gladioli*	**70**	**80**	**n**	**+**
239	BCC1556	07-3	ENVI	*B. gladioli*	**70+**	**36.6**	**n**	**-**
240	BCC0507	PF (#428)	CF	*B. gladioli*	**29.7**	**80.3**	**n**	**-**
241	BCC1623	AU17121	CF	*B. gladioli*	**35 (faint)**	**66.3**	**n**	**+**
242	BCC1619	AU10372	CF	*B. gladioli*	**15 (faint)**	**27.3**	**n**	**-**
243	BCC1317	41	ENVI	*B. gladioli*	**25**	**82.3**	**n**	**+++**
244	BCC0772	LMG 14190, ATCC 29195	ENV	*B. glathei*	**0**	**0**	**n**	**-**
245	BCC0773	LMG 2196, ATCC 33617	ENV	*B. glumae*	**0**	**52.3**	**n**	**-**
246	BCC0774	LMG 18924, ATCC 700544	ENV	*B. graminis*	**0**	**0**	**n**	**-**
247	BCC1612	LMG 20598	ENV	*B. hospital*	**0**	**0**	**n**	**-**
248	BCC0775	LMG 19447, ATCC 700977	ENV	*B. kuruiensis*	**7.7 (faint)**	**0**	**n**	**-**
249	BCC0803	LMG 22485 ATCC 17660	ENV	*B. lata*	**9.3**	**9**	**n**	**+++**
250	BCC1406	(6 = send number)	ENVI	*B. lata*	**22 (faint)**	**0**	**n**	**-**
251	FC0436	ATCC 17769 LMG 6992	ENV	*B. lata*	**16.6**	**23.5**	**n**	**+++**
252	BCC0402	B5	ENV	*B. lata*	**0**	**0**	**n**	**+++**
253	CEP0085	ATCC 17460, LMG 6863	ENV	*B. lata*	**21.3**	**28**	**n**	**+++**
254	BCC0400	B1	ENV	*B. lata*	**0**	**0**	**n**	**++**
255	CEP1061	patient 24; CEP1061	CF	*B. lata*	**0**	**0**	**n**	**+**
256	BCC0404	B14	ENV	*B. lata*	**10**	**12**	**n**	**+++**
257	BCC1296	16	ENVI	*B. lata*	**0**	**0**	**n**	**++**
258	BCC1287	6	ENVI	*B. lata*	**0**	**0**	**n**	**-**
259	BCC1282	1	ENVI	*B. lata*	**0**	**0**	**n**	**++**
260	BCC1625	LMG 24064	CF	*B. latens*	**0**	**0**	**n**	**-**
261	BCC1626	LMG 24264	CF	*B. latens*	**34.3**	**45.6**	**n**	**-**

262	BCC0483	AU0553	CF	*B. metallica*	**21.3**	**20.1**	**n**	**-**
263	BCC1629	LMG 24068	CF	*B. metallica*	**21.6**	**0**	**n**	**+**
264	BCC1606	LMG 25256	ENV	*B. mimosarum*	**0**	**9.6 (faint)**	**n**	**-**
265	C0514	KE1; CO514	CF	*B. multivorans*	**0**	**28 (faint)**	**0**	**-**
266	CEP0049	LMG 18823;	MUT	*B. multivorans*	**0**	**0**	**n**	**-**
267	BCC0904	93-N331	NON	*B. multivorans*	**0**	**0**	**5.3 faint**	**-**
268	CEP0992	EJ; CEP0992	CF	*B. multivorans*	**0**	**35 (faint)**	**0**	**-**
269	BCC0375	AU0607	CF	*B. multivorans*	**0**	**35 (faint)**	**0**	**-**
270	BCC0390	LMG 18825;	CF	*B. multivorans*	**0**	**40 (faint)**	**0**	**-**
271	C6935	C1; C6935	CF	*B. multivorans*	**0**	**39 (faint)**	**0**	**-**
272	CEP0109	JT; CEP0178	CGD	*B. multivorans*	**0**	**39 (faint)**	**0**	**-**
273	C5393	LMG 18822	CF	*B. multivorans*	**0**	**30 (faint)**	**0**	**-**
274	BCC1420	C1978	CF	*B. multivorans*	**0**	**0**	**n**	**-**
275	CEP0144	ATCC 17616; LMG17588	ENV	*B. multivorans*	**0**	**30 (faint)**	**0**	**-**
276	CEP0781	LMG 16660	CF	*B. multivorans*	**0**	**32 (faint)**	**0**	**-**
277	CEP0602	YP1; CEP0602	CF	*B. multivorans*	**0**	**37 (faint)**	**0**	**-**
278	CEP0600	(J BURNS); CEP0600	NON	*B. multivorans*	**0**	**0**	**0**	**-**
279	FC445R	LMG 13010	CF	*B. multivorans*	**0**	**0**	**0**	**-**
280	BCC0493	BELF 1	CF	*B. multivorans*	**0**	**0**	**0**	**-**
281	FC0328	FC0328	CF	*B. multivorans*	**0**	**24 (faint)**	**0**	**-**
282	FC0647	BP102	ENV	*B. multivorans*	**0**	**30 (faint)**	**14.6 faint**	**-**
283	CEP0494	JM; CEP0494	CF	*B. multivorans*	**0**	**38 (faint)**	**0**	**-**
284	CEP0445	4F/MATLOW	CF	*B. multivorans*	**0**	**38 (faint)**	**0**	**-**
285	FC0762	LMG 16665	NON	*B. multivorans*	**0**	**36 (faint)**	**0**	**-**
286	CEP0935	KH; CEP0935	NON	*B. multivorans*	**0**	**35 (faint)**	**0**	**-**
287	FC0442	GM; FC0442	CGD	*B. multivorans*	**0**	**38 (faint)**	**0**	**-**
288	CEP0181	C1664/GOVAN	ENVH	*B. multivorans*	**0**	**0**	**0**	**-**
289	FC0147	LMG 18824; JTC	CGD	*B. multivorans*	**0**	**0**	**0**	**-**
290	BCC0702	IST453	CF	*B. multivorans*	**0**	**0**	**0**	**-**
291	FC0784	15922-1	ENV	*B. multivorans*	**0**	**35 (faint)**	**0**	**-**
292	BCC1605	LMG 23618	ENV	*B. oklahomensis*	**22**	**40.6**	**n**	**+**
293	BCC0776	LMG 2247	ENV	*B. phenazinium*	**25**	**60.6**	**n**	**+++**
294	BCC1611	LMG 22037	ENV	*B. phenoliruptrix*	**0**	**8.6 (faint)**	**n**	**-**
295	BCC1607	LMG 21445	ENV	*B. phymatum*	**0**	**11.3 (faint)**	**n**	**-**
296	BCC1604	LMG 22487	ENV	*B. phytofirmans*	**0**	**0**	**n**	**-**
297	BCC0777	LMG 9035, ATCC 43733	ENV	*B. plantarii*	**14.3 (faint)**	**82.6**	**n**	**++**
298	BCC1020	MDIII-B-65	ENV	*B. pyrocinia*	**13.7**	**12**	**n**	**++**
299	BCC1346	HI3647	ENV	*B. pyrocinia*	**18.3**	**19.5**	**n**	**-**
300	BCC1104	MVP-C2-46	ENV	*B. pyrocinia*	**20.3**	**21**	**n**	**+**
301	FC0451	LMG 14191-T	ENV	*B. pyrocinia*	**0**	**17**	**n**	**-**
302	BCC0986	MVPC1-31	ENV	*B. pyrocinia*	**24.6**	**15**	**n**	**++**
303	BCC0662	R-13061; (J2542)	ENV	*B. pyrocinia*	**0**	**0**	**n**	**-**
304	BCC0734	ES0209	ENV	*B. pyrocinia*	**29 (faint)**	**11**	**n**	**+**
305	FC0433	ATCC 39277; LMG 21822	ENV	*B. pyrocinia*	**0**	**16**	**n**	**-**
306	BCC0490	ES0153; R-13545	ENV	*B. pyrocinia*	**11 (faint)**	**19 (faint)**	**n**	**-**
307	BCC0980	MDI-43	ENV	*B. pyrocinia*	**17**	**0**	**n**	**+**
308	BCC0979	MCII-94	ENV	*B. pyrocinia*	**19.5**	**0**	**n**	**+**
309	BCC0488	ES0128; R-13543	ENV	*B. pyrocinia*	**21.8 (faint)**	**36 (faint)**	**n**	**-**
310	BCC0735	AU2419	CF	*B. pyrocinia*	**12.6**	**10 (faint)**	**n**	**++**
311	BCC0476	LMG 21823	ENV	*B. pyrocinia*	**12.8 (faint)**	**13.5**	**n**	**-**
312	BCC1348	HI3642	ENV	*B. pyrocinia*	**0**	**0**	**n**	**-**
313	BCC1364	AU5468	CF	*B. pyrocinia*	**19.3**	**26**	**n**	**-**
314	BCC0778	LMG 19450	ENV	*B. sacchari*	**0**	**0**	**n**	**-**
315	BCC1627	LMG 24067	CF	*B. seminalis*	**20.3**	**0**	**n**	**-**
316	BCC1628	LMG 19587	ENV	*B. seminalis*	**12.6**	**0**	**n**	**-**
317	BCC0479	HI-2482	ENV	*B. stabilis*	**0**	**0**	**25**	**-**

							(faint)	
318	BCC0717	P1-5	CF	*B. stabilis*	**0**	**0**	**28.6 (faint)**	**-**
319	BCC0418	CRUPI	NON	*B. stabilis*	**0**	**0**	**12.6 (faint)**	**-**
320	CEP0104	ATCC 35254	ENVH	*B. stabilis*	**0**	**0**	**21.3(faint)**	**-**
321	BCC1342	AU6735	CF	*B. stabilis*	**0**	**7**	**0**	**-**
322	FC0367	LMG 14294	CF	*B. stabilis*	**0**		**n**	**-**
323	FC0472	LMG 14086;	ENVH	*B. stabilis*	**0**	**0**	**20.3**	**-**
324	BCC1325	51	ENVI	*B. stabilis*	**0**	**0**	**0**	**-**
325	BCC1347	AU9640	CF	*B. stabilis*	**0**	**0**	**25 (faint)**	**-**
326	CEP0928	ALBUTEROL CEP0928	ENVH	*B. stabilis*	**0**	**7.5**	**25 (faint)**	**-**
327	FC0362	LMG 07000	NON	*B. stabilis*	**0**	**0**	**25 (faint)**	**-**
328	BCC1308	30	ENVI	*B. stabilis*	**0**	**0**	**25**	**-**
329	FC0779	LMG 18888	NON	*B. stabilis*	**18.3 (faint)**	**0**	**n**	**-**
330	BCC1352	AU10096	CF	*B. stabilis*	**0**	**0**	**25.3 (faint)**	**-**
331	CEP0103	ATCC 27515	NON	*B. stabilis*	**0**	**0**	**25 (faint)**	**-**
332	BCC0899	88-N312	NON	*B. stabilis*	**0**	**0**	**20**	**-**
333	BCC0957	146-N491	NON	*B. stabilis*	**0**	**0**	**0**	**-**
334	BCC0828	17-474	CF	*B. stabilis*	**0**	**0**	**12.6 (faint)**	**-**
335	BCC1609	LMG 20594	ENV	*B. terricola*	**20.7 (faint)**	**15.6 (faint)**	**n**	**-**
336	BCC1608	LMG 22274	ENV	*B. tropica*	**0**	**12.6 (faint)**	**n**	**-**
337	BCC1610	LMG 21444	ENV	*B. uberum*	**0**	**10.6 (faint)**	**n**	**-**
338	BCC1603	LMG 20358	ENV	*B. ubonensis*	**17 (faint)**	**14.3**	**n**	**-**
339	FC0353	FC0353	CF	*B. vietnamiensis*	**0**	**0**	**n**	**++**
340	CEP0998	BBG1222	ENV	*B. vietnamiensis*	**36.0**	**45.5**	**n**	**+**
341	FC0369	LMG 10929	ENV	*B. vietnamiensis*	**0**	**52**	**n**	**+**
342	FC0441	LMG 18836; FC0441	CGD	*B. vietnamiensis*	**0**	**35 (faint)**	**n**	**-**
343	FC0656	ATCC 53617; LMG 22486	ENV	*B. vietnamiensis*	**0**	**0**	**n**	**-**
344	CEP0626	CLO; CEP0626	CF	*B. vietnamiensis*	**0**	**28.6**	**n**	**++**
345	FC0769_am	LMG 16232; FC0466	CF	*B. vietnamiensis*	**0**	**0**	**n**	**-**
346	FC0654	CRE-7	ENV	*B. vietnamiensis*	**0**	**42 (faint)**	**n**	**++**
347	CEP0255	MO1; CEP0255	CF	*B. vietnamiensis*	**0**	**43 (faint)**	**n**	**++**
348	BCC1409	JW13.2a	ENVI	*B. vietnamiensis*	**20.3**	**51.5**	**n**	**+**
349	CEP0160	J1702/GOVAN	ENVH	*B. vietnamiensis*	**0**	**0**	**n**	**-**
350	CEP0040	LMG 18835	CF	*B. vietnamiensis*	**0**	**43 (faint)**	**n**	**++**
351	CEP0047	RJ2; CEP0047	NON	*B. vietnamiensis*	**0**	**47**	**n**	**+**
352	CEP0192	J1742/GOVAN	NON	*B. vietnamiensis*	**0**	**0**	**n**	**+**
353	BCC1408	JW13.1a	ENVI	*B. vietnamiensis*	**0**	**49.5**	**n**	**-**
354	BCC1309	31	ENVI	*B. vietnamiensis*	**21.3**	**36.5**	**n**	**-**
355	BCC1301	23	ENVI	*B. vietnamiensis*	**41.6**	**11**	**n**	**-**
356	BCC1172	D0099	ENV	*B. vietnamiensis*	**0**	**0**	**n**	**-**
357	BCC0657	LB400	ENV	*B. xenovorans*	**0**	**0**	**n**	**-**
358	BCC1302	24	ENVI	BCC group Kc?	**0**	**0**	**n**	**++**
359	BCC0475	BC003	ENV	BCC4	**32.3**	**12**	**n**	**-**
360	BCC0405	T21	ENV	BCC4	**13.3 (faint)**	**22.5**	**n**	**++**
361	CEP0152	LMG21824	CF	BCC4	**12**	**17.5**	**n**	**++**
362	BCC1017	MDII-124riz	ENV	BCC5	**9**	**12**	**n**	**-**
363	BCC0419	MC II 141	ENV	BCC5	**9.6**	**9**	**n**	**-**
364	BCC0397	R-10733t1	NON	BCC5	**9**	**10**	**n**	**-**
365	BCC1042	MVP-C2-53	ENV	BCC5	**9.6**	**8 (faint)**	**n**	**-**
366	BCC0474	NI (#294)	CF	BCC5	**14.6**	**10**	**n**	**-**

					(faint)			
367	BCC1047	MVP-C2-84	ENV	BCC6	**24.6**	**20**	**n**	**-**
368	BCC0985	MVPC1-13	ENV	BCC6	**17**	**0**	**n**	**-**
369	BCC0976	MVPC2-6	ENV	BCC6	**20**	**7**	**n**	**-**
370	CEP0178	C1518/GOVAN; CEP0178	CF	BCC6	**9.3**	**0**	**n**	**++**
371	BCC1013	MDII-144pott	ENV	BCC6	**22.3**	**10**	**n**	**+**
372	BCC1005	MDI-14a	ENV	BCC6	**23.3**	**10**	**n**	**+**
373	BCC1038	MVP-C1-79	ENV	BCC6	**23.3**	**11.5**	**n**	**+**
374	BCC1023	MDIII-P-115	ENV	BCC6	**22.3**	**12**	**n**	**+**
375	BCC1026	MDIII-T-301	ENV	BCC6	**22.6**	**13.5**	**n**	**+**
376	BCC1030	MVP-C1-16	ENV	BCC6	**23.6**	**12**	**n**	**-**
377	BCC1191	D1443	CF	BCC8	**0**	**0**	**n**	**-**
378	C8502	WK1	CF	*Burk. sp, not BCC*	**0**	**9.6 (faint)**	**n**	**-**
379	BCC1387	LMG 1226		*D. acidivorans*	**0**	**0**	**n**	**-**
380	BCC0671	IST422	CF	NOT BCC	**0**	**10.6**	**n**	**-**
381	BCC0498	CARBONARO	CF	NOT BCC	**17.3**	**0**	**n**	**-**
382	BCC0696	IST447	CF	NOT BCC	**0**	**0**	**n**	**-**
383	BCC0690	IST442	CF	NOT BCC	**15 (faint)**	**0**	**n**	**-**
384	BCC0677	IST429	CF	NOT BCC	**0**	**0**	**n**	**-**
385	BCC0454	A2	ENV	NOT BCC	**48**	**45.3**	**n**	**++**
386	BCC0667	IST417	CF	NOT BCC	**0**	**0**	**n**	**-**
387	BCC0473	SD	CF	NOT BCC	**7.7 (faint)**	**0**	**n**	**-**
388	BCC0471	ZC1	NON	NOT BCC	**0**	**21.3**	**n**	**-**
389	BCC0469	F6 98150	ENV	NOT BCC	**0**	**0**	**n**	**-**
390	BCC0440	F6 99173	ENV	NOT BCC	**0**	**0**	**n**	**-**
391	BCC0502	PM 16/04)	CF	NOT BCC	**7.3 (faint)**	**0**	**n**	**-**
392	BCC0420	ME	CF	NOT BCC	**0**	**0**	**n**	**-**
393	BCC0457	CS Pg	CF	NOT BCC	**0**	**7.6**	**n**	**-**
394	BCC0689	IST441	CF	NOT BCC	**0**	**40.6**	**n**	**-**
395	BCC0421	DV	CF	NOT BCC	**0**	**0**	**n**	**-**
396	BCC0465	A6	ENV	NOT BCC	**0**	**0**	**n**	**-**
397	BCC0435	IK 7/12	CF	NOT BCC	**0**	**0**	**n**	**-**
398	BCC0422	A5	ENV	*P. aeruginosa*	**38**	**64.3**	**n**	**+++**
399	BCC0432	A4	ENV	*P. aeruginosa*	**31.7**	**47.6**	**n**	**++**
400	BCC0438	A1	ENV	*P. aeruginosa*	**36**	**50.6**	**n**	**+++**
401	BCC1388	LMG 1794 Type strain	ENV	*P. fluorescens*	**21.7 (faint)**	**0**	**n**	**-**
402	BCC1389	LMG 2257	ENV	*P. putida*	**0**	**0**	**n**	**-**
403	BCC1390	LMG 11199 Type strain		*P. stutzeri*	**14.7**	**0**	**n**	**-**
404	FC0330	40	CF	*Pand. Pulmonicola*	**13 (faint)**	**0**	**n**	**-**
405	CEP0633	PATIENT W	CF	*Pand. apista*	**0**	**0**	**n**	**-**
406	C8029	K2	CF	*Pand. pnomenusa*	**0**	**0**	**n**	**-**
407	C7351	KZ1	CF	*Pand. pnomenusa*	**0**	**0**	**n**	**-**
408	CEP0734	AU0012	CF	*Pand. sputorum*	**0**	**0**	**n**	**-**
409	CEP0687	BC259-B1	CF	*Pand. sputorum*	**0**	**0**	**n**	**-**
410	C4964	QG	CF	*Pand. sputorum*	**0**	**0**	**n**	**-**
411	BCC1391	LMG 6866 Type strain	CLIN	*R. mannitolytica*	**0**	**0**	**n**	**-**
412	BCC1392	LMG 5942 Type strain	CLIN	*R. pickettii*	**13 (faint)**	**0**	**n**	**-**
413	CEP0115	C1739/GOVAN	CF	*R. pickettii*	**12 (faint)**	**0**	**n**	**-**
414	BCC1393	LMG 22203 Type strain	CLIN	*S. aureus*	**0**	**0**	**n**	**0**
415	BCC1394	LMG 958	CLIN	*S. maltophilia*	**0**	**0**	**n**	**-**
416	BCC0809	NW stain, H044220140	CLIN	*A. baumannii*	**0**	**0**	**n**	**-**
417	BCC0811	T strain, 173	CLIN	*A. baumannii*	**19.7**	**0**	**n**	**-**
418	BCC0810	SE clone,	CLIN	*A. baumannii*	**20**	**0**	**n**	**-**

		H043260547			**(faint)**			
419	BCC0808	OXA-23	CLIN	*A. baumannii*	**0**	**0**	**n**	**-**
420	BCC0807	OXA-23 clone, H074200542	CLIN	*A. baumannii*	**13 (faint)**	**0**	**n**	**-**
421	BCC1386	LMG 1863		*A. xylosoxidans*	**20 (faint)**	**0**	**n**	**-**

APPENDIX B

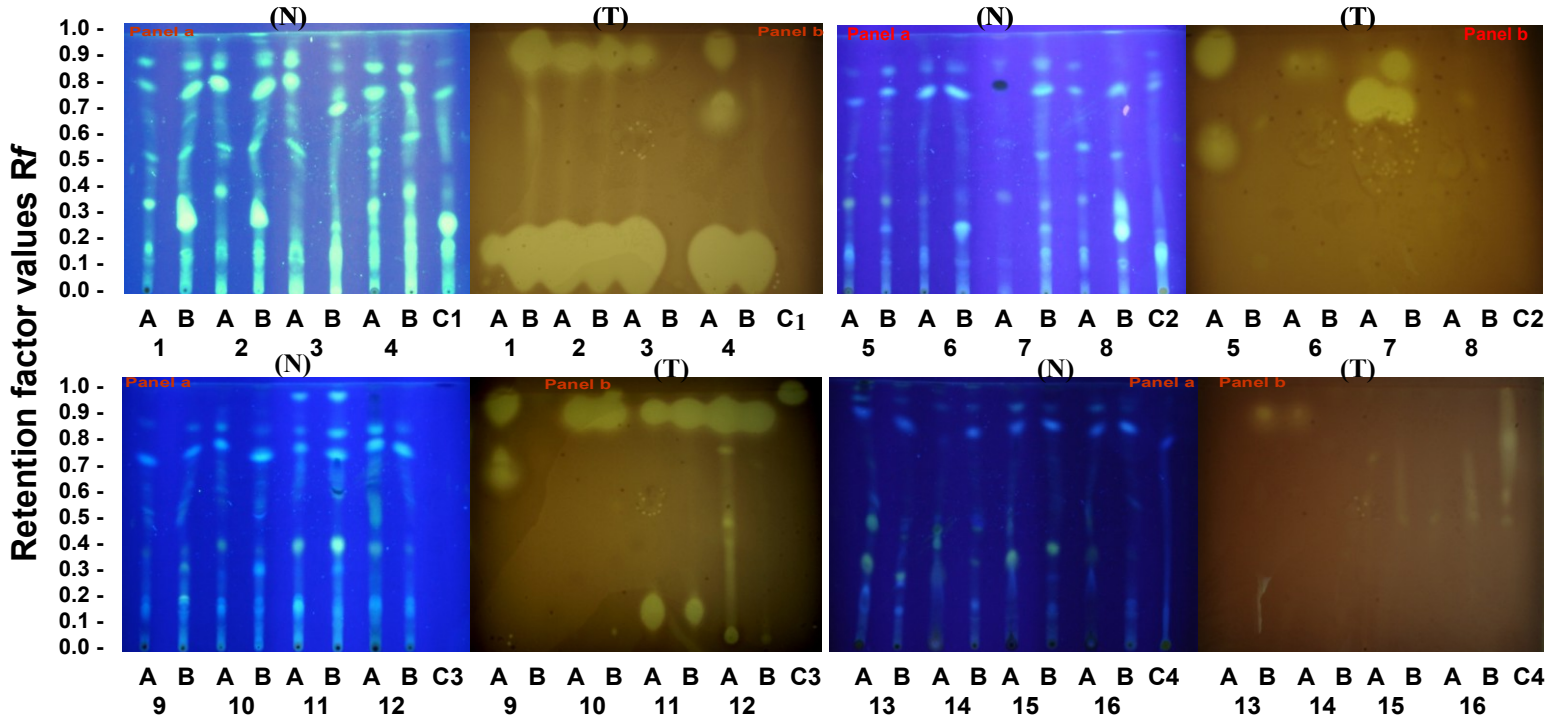

Figure B. 1 Fractionation and detection of anti-candidal activity of 16 *Burkholderia* crude resin extracts using TLC and ethyl acetate methanol water (20:1:0.5).

Crude resin extracts of 16 Burkholderia, pyrrolnitrin (Pyr) Sabouraud agar (SA) and Basal salts agar (BSM) were spotted onto TLC plates in duplicate then fractionated using ethyl acetate methanol water (20:1:0.5). One set of developed TLC plates was visualized using UV at 366 nm (Panel N).The other set was used for bioautography assays, the plate was overlaid with soft Isosensitest agar seeded with C. albicans, after incubation of plates at room temperature for 3 h and then 24 h at 37°C, the anti-candidal activity of different compounds are clearly visible as a clear zone on the red background of Candida cells which have grown and reduced the tetrazolium chloride metabolic indicator (Panel T).A; Sabouraud Agar, B, Basal Salt Agar, 1; B. ambifaria Bcc0276, 2; B. ambifaria Bcc0410, 3; B. ambifaria Bcc0478, 4; B. ambifaria Bcc1599, 5; B. lata Bcc1265, 6; B. ambifaria Bcc0372, 7; B. ambifaria Bcc0207, 8; B. ambifaria Bcc0193, 9; B. vietnamiensis Bcc1409, 10; B. contaminants Bcc1315, 11; B. anthina Bcc1350, 12; B. cepacia Bcc0233, 13; B. cenocepacia Bcc019, 14; B. cenocepacia Bcc0165 15; B. lata Bcc0803, 16; B. pyrrocinia Bcc0171, C1; BSM, C2; SA, C3; Pyrrolnitrin.

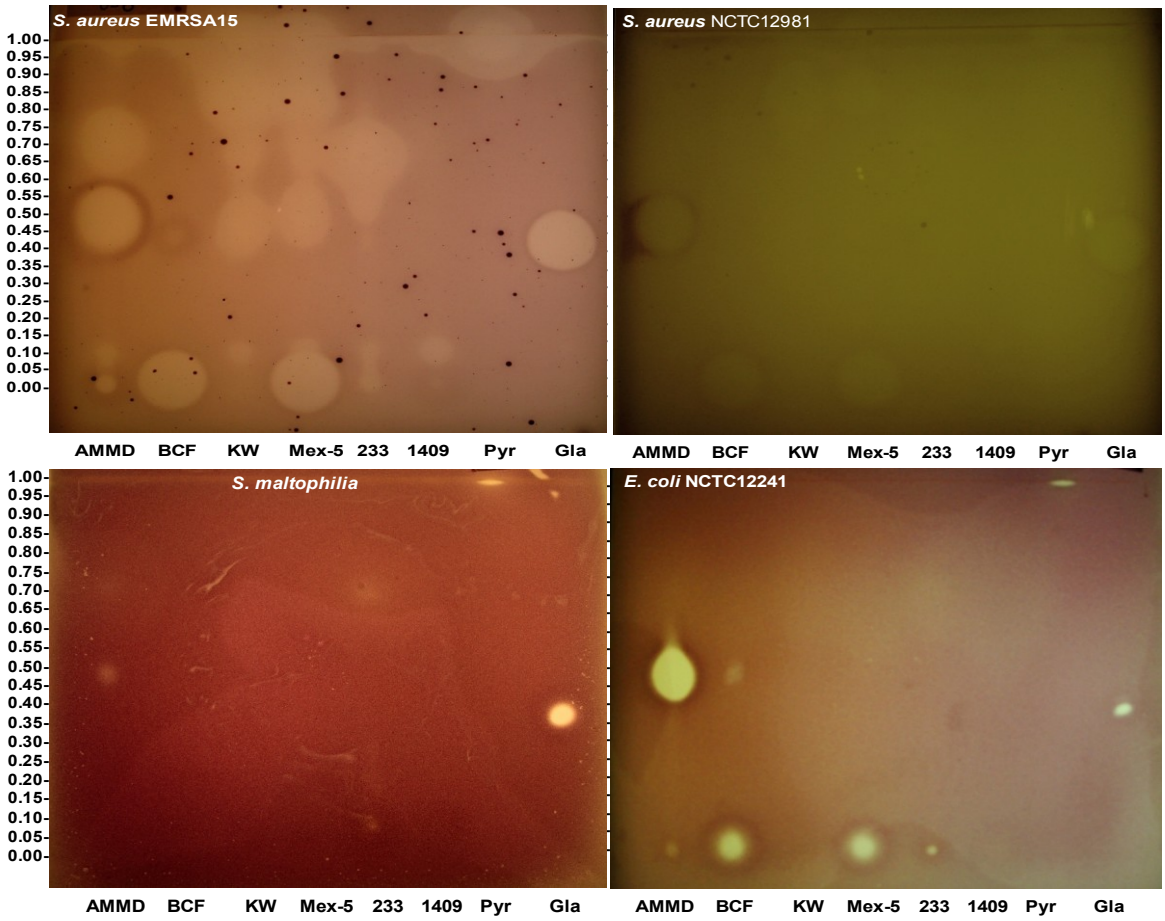

Figure B. 2 Detection of antibacterial agents in *Burkholderia* crude resin extracts.

Crude resin extracts of 6 Burkholderia, pyrrolnitrin and pure gladiolin were spotted onto TLC plates in duplicate then fractionated using ethyl acetate methanol water (20:1:0.5). One set of developed TLC plates was visualized using UV at 366 nm. The other set was used for bioautography assays, the plate was overlaid with soft Isosensitest agar seeded with gram negative bacteria, after incubation of plates at room temperature for 3 h and then 24 h at 37°C, the antibacterial activity of different compounds are clearly visible as a clear zone on the red background of bacterial cells which have grown and reduced the tetrazolium chloride metabolic indicator. AMMD, B. ambifaria Bcc0207; BCF, B. ambifaria Bcc0250; KW, B. ambifaria Bcc1256; MEX-5, B. ambifaria Bcc1599; 233, B. lata Bcc0233; 1409, B. vietnamiensis Bcc1409; Pyr, pure pyrrolnitrin; Gla, pure gladiolin. The overlaid bacteria were: A, A. baumannii; B, P. fluorescens; C, S. maltophilia ; D, E. coli., B, S. aureus Eagles; C, S. aureus NCTC12981.

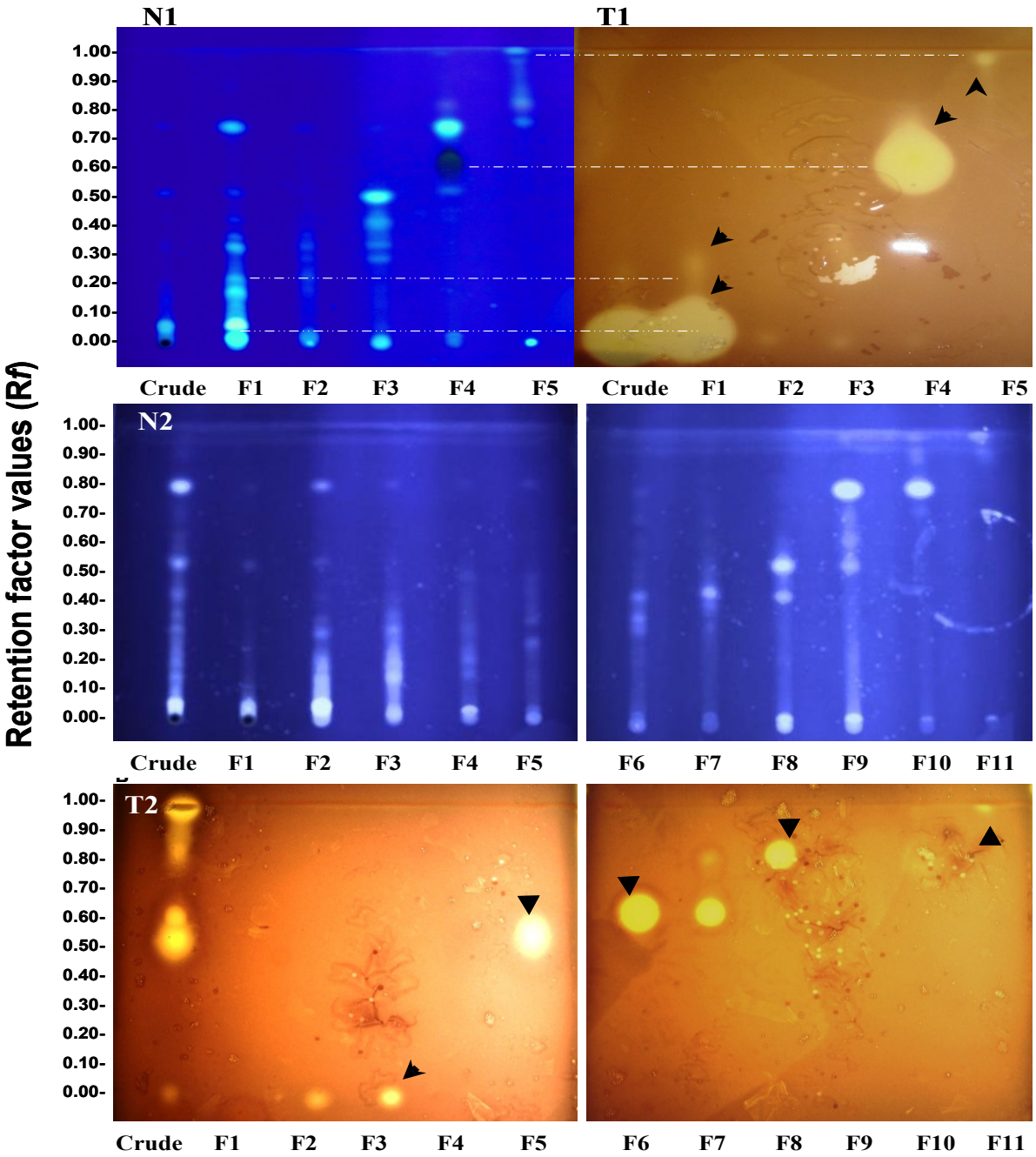

Figure B. 3 Profile of *B. ambifaria* BCF and KW metabolites fractionated using silica gel preparative TLC.

Semi-pure fractions fractionated from cured resin extract and cured resin extract of B. ambifaria BCF (fractions 1 to 5) and B. ambifaria KW (fractions 1 to 11) were spotted into TLC plates in duplicate. One set of TLC plates was developed with ethyl acetate: methanol: water (20:1:0.5) (panels A1 and A2). The other set was overlaid with soft Isosensitest agar seeded with C. albicans. After incubation of plates at room temperature for 3 h and then 24 h at 37°C, the anti-candidal activity of different compounds was visible as a clear zone on the red background of Candida cells which had grown and reduced the tetrazolium chloride metabolic indicator (panels B1 and B2). Arrows indicated the location of active semi-pure metabolites.

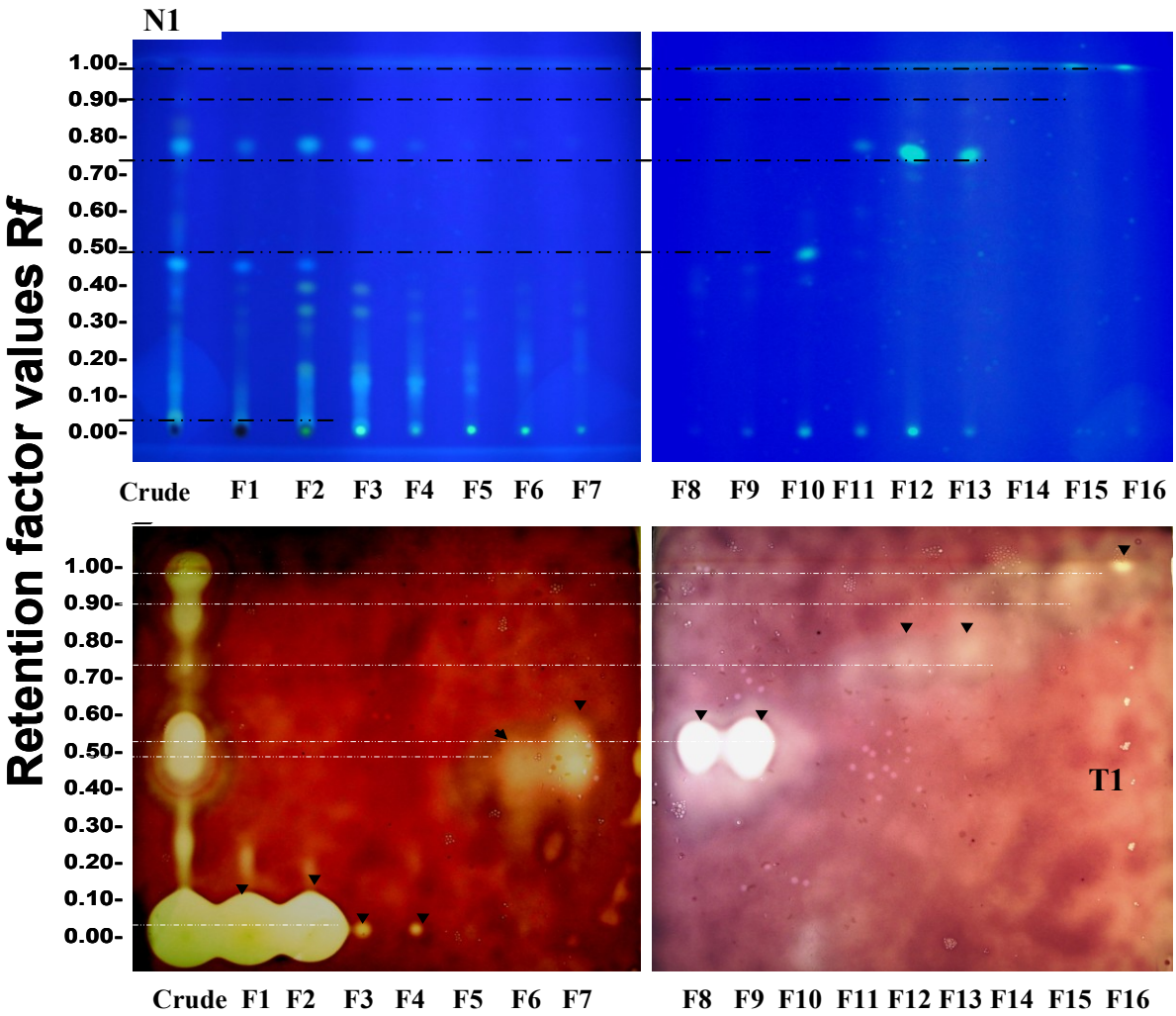

Figure B. 4 Profile of *B. ambifaria* Mex-5 metabolites fractionated using silica gel preparative TLC.

Semi-pure fractions (Fraction 1 to fraction 16) fractionated from cured resin extract and cured resin extract of B. ambifaria Mex-5 were spotted into TLC plates in duplicate. One set of TLC plates was developed with ethyl acetate: methanol: water (20:1:0.5) (panel T). The other set was overlaid with soft Isosensitest agar seeded with C. albicans. After incubation of plates at room temperature for 3 h and then 24 h at 37°C, the anti-candidal activity of different compounds was visible as a clear zone on the red background of Candida cells which have grown and reduced the tetrazolium chloride metabolic indicator (panel N). Arrows indicate the location of active semi-pure metabolites.

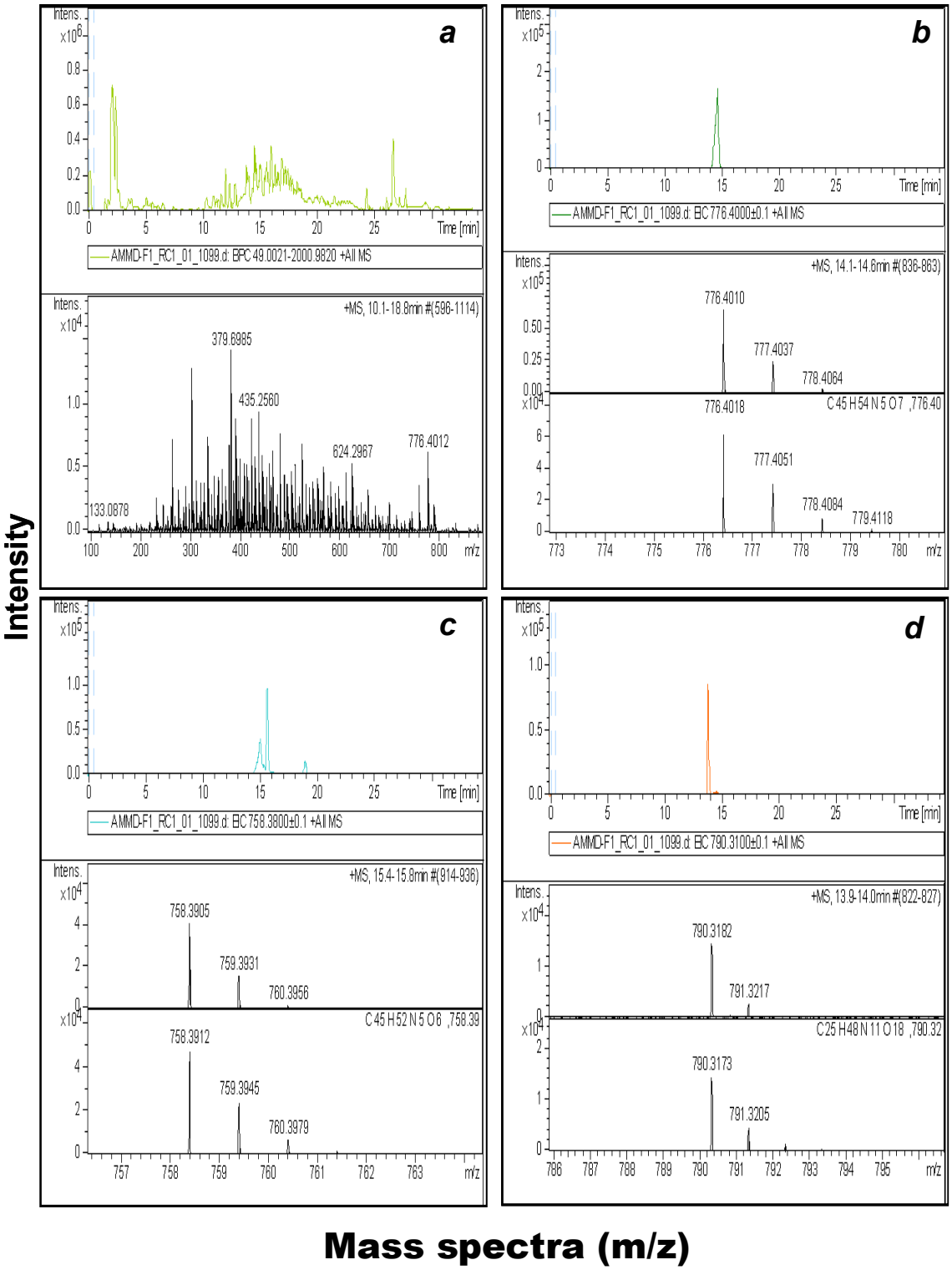

Figure B. 5 The mass spectra of *B. ambifaria* AMMD fraction 1 (Rf 0.01).

(a) Polyethylene glycol (PEG); (b) Unknown peptide and (c and d) plasticizer.

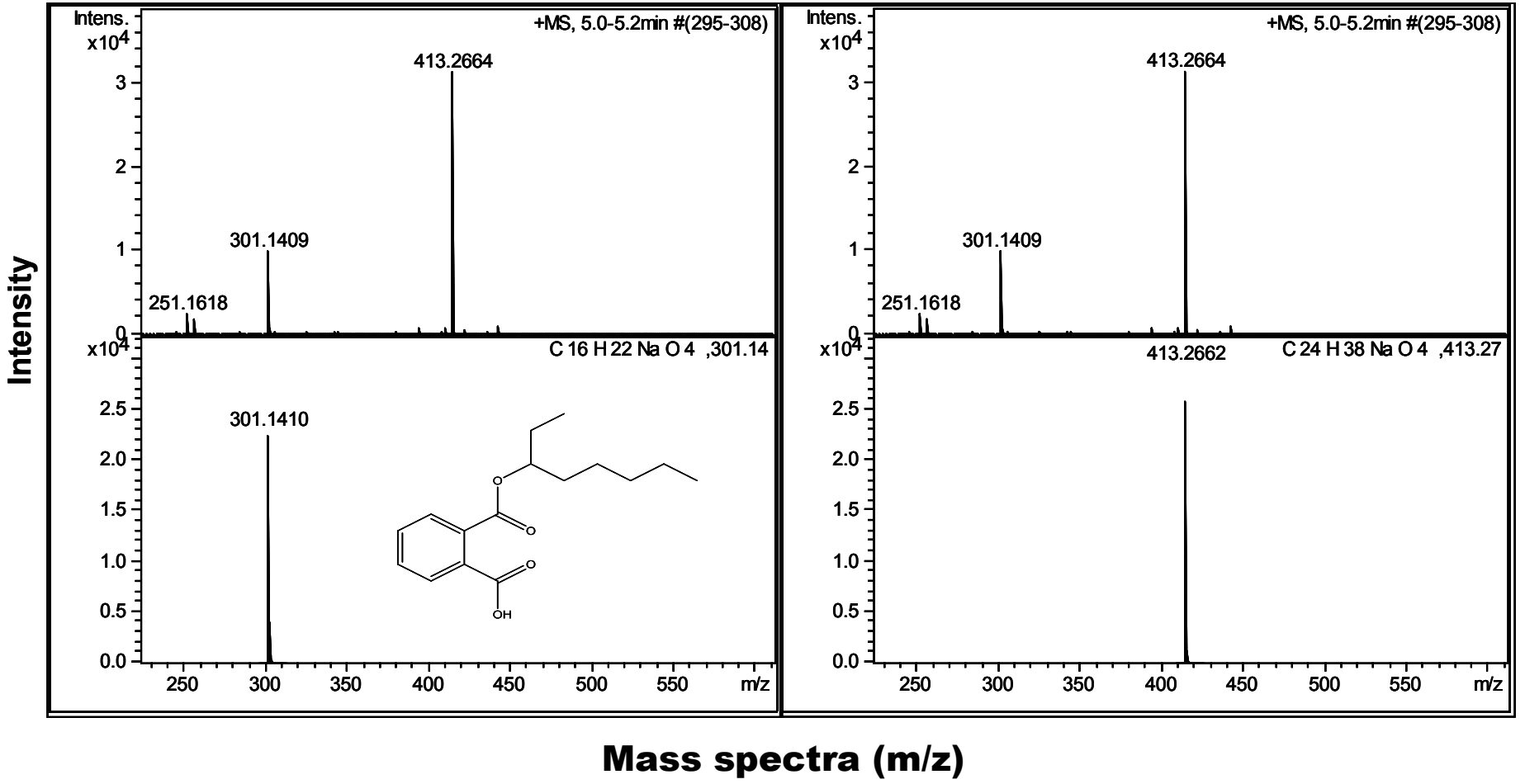

Figure B. 6 Mass spectra data for *B. ambifaria* AMMD fraction 7 (Rf 0.98).

Results indicated that fraction 7 is dominated by different of plasticizer. The chemical structure shown on right panel is plasticizer.

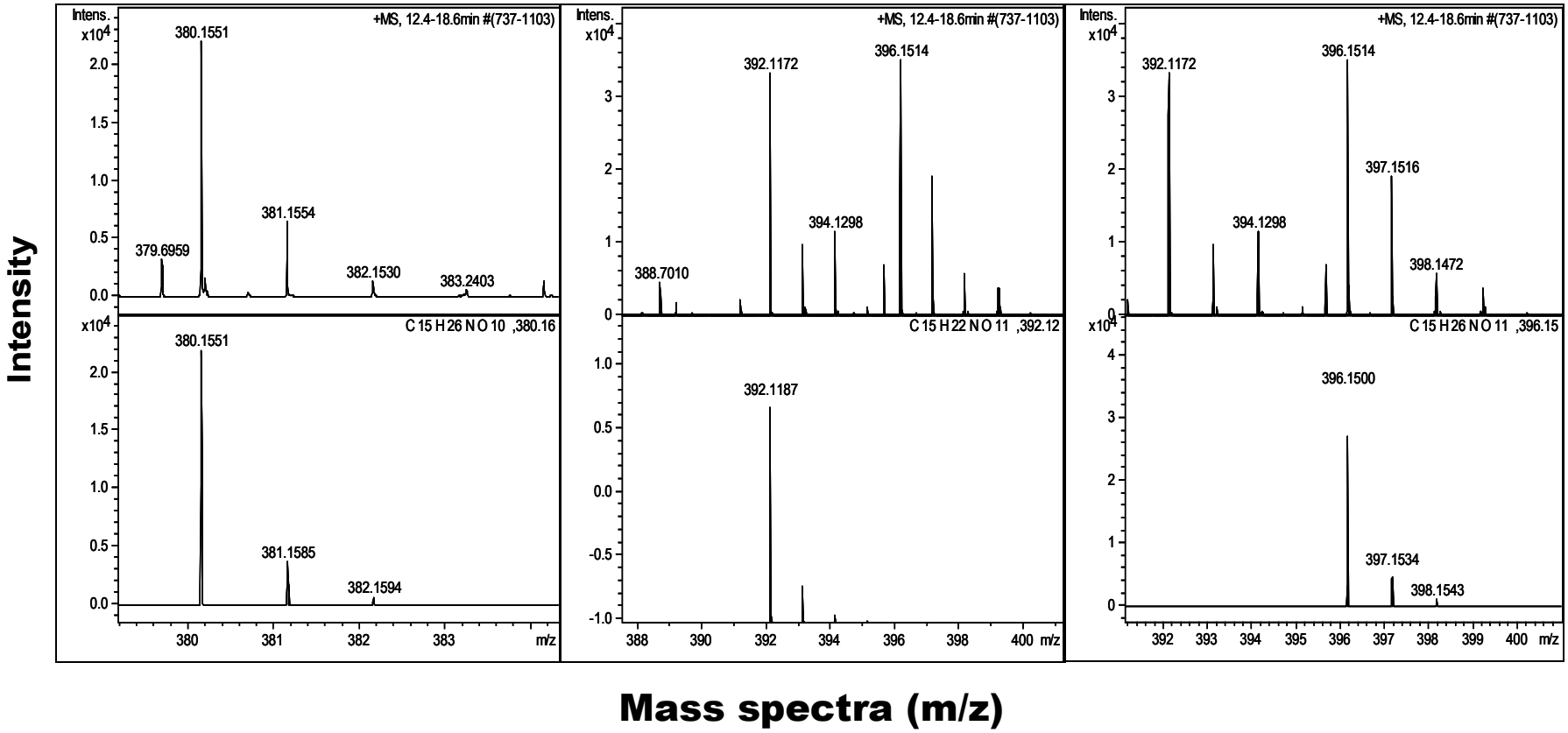

Figure B. 7 Mass spectra data for *B. ambifaria* KW fraction 1.

Results indicated that fraction 1 was dominated by many of unknowns of un-identified compounds (380.16, 392.12 and 396.15 m/z).

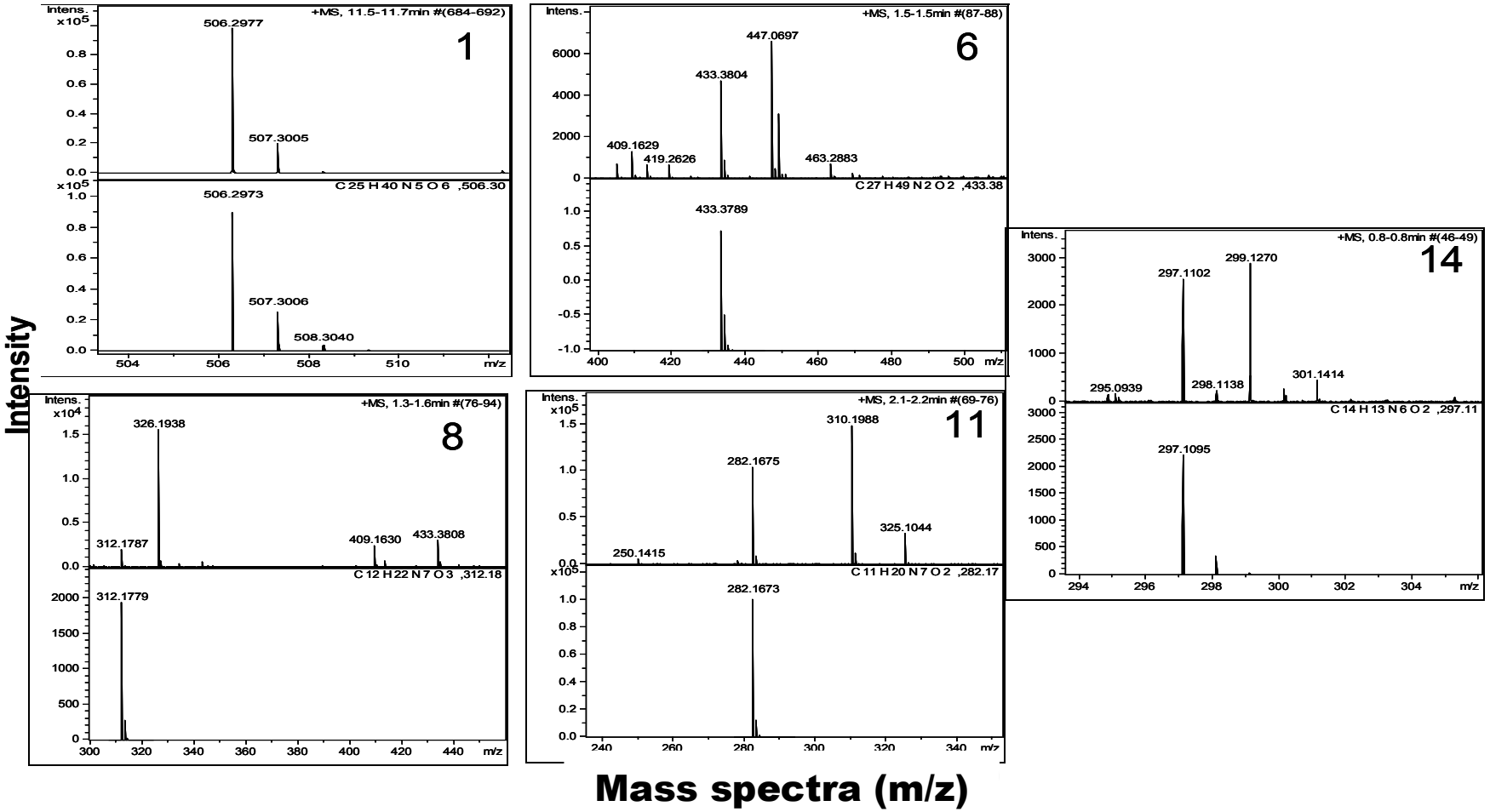

Figure B. 8 Mass spectra data of *B. ambifaria* Mex-5.

The results indicated that Mex-5 produced many of un-identified compounds. Fraction 1, 506.29; fraction 6, 433.37 m/z; fraction 8, 312.17 m/z; fraction 11, 282.16 m/z ;and fraction 14, 297.1 m/z.

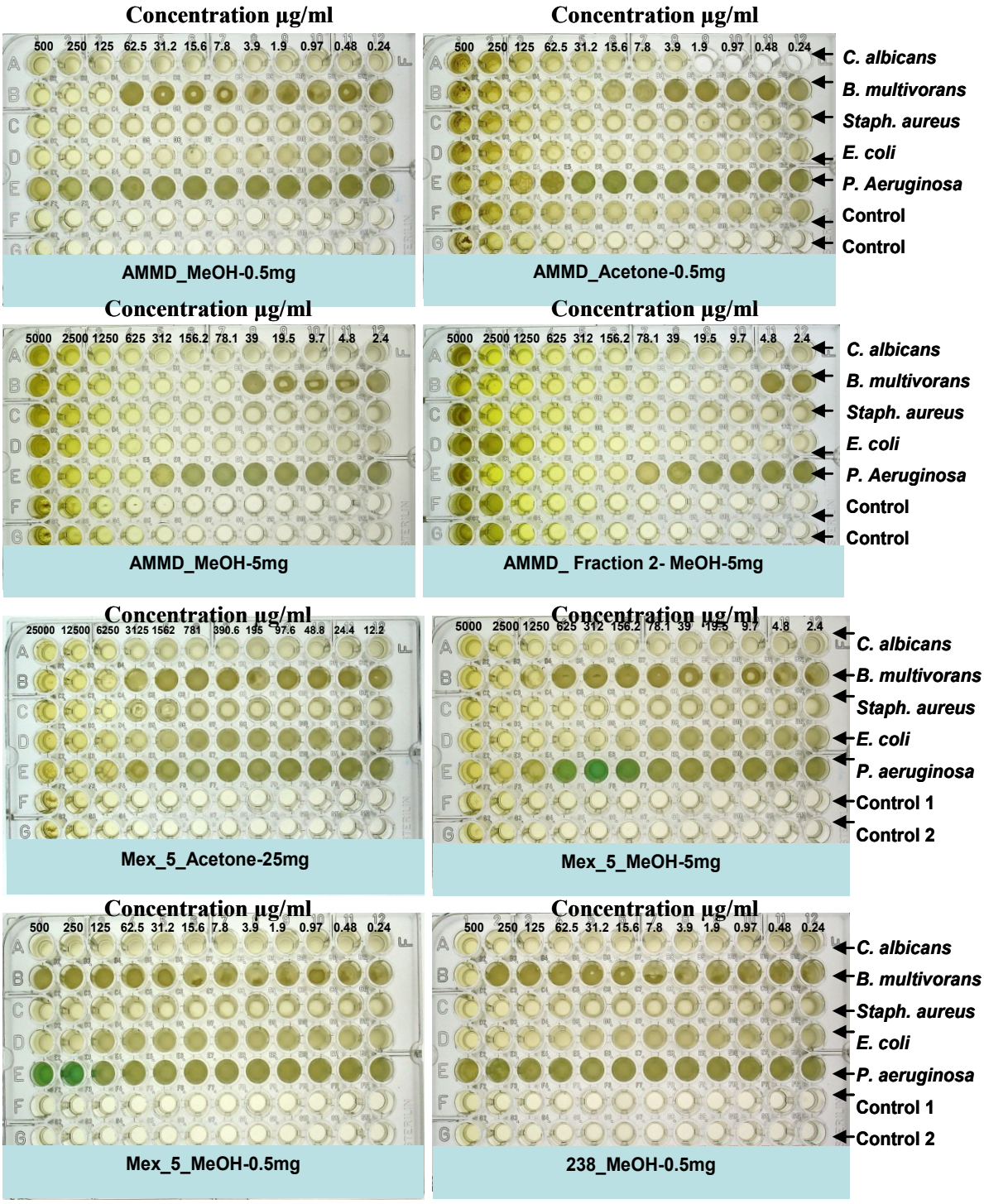

Figure B. 9 The broth dilution assay used to determine the activity of *Burkholderia* antimicrobial extracts.

The minimum inhibitory concentrations (µg/ml) of *B. ambifaria* AMMD, BCF, KW and Mex_5, and *B. gladioli* extracts against *C. albicans* (Sc5314), *Staphylococcus aureus* (NCTC12981), *B. multivorans* (ATCC17210), *Escherichia coli* (NCTC12241) and *Pseudomonas aeruginosa* (NCTC12903). Control 1 was sterilised BSM-G medium, Control 2 was *Burkholderia* extracts alone, MeOH; was crude resin extract and Acetone; was crude acetone extract.

APPENDIX C

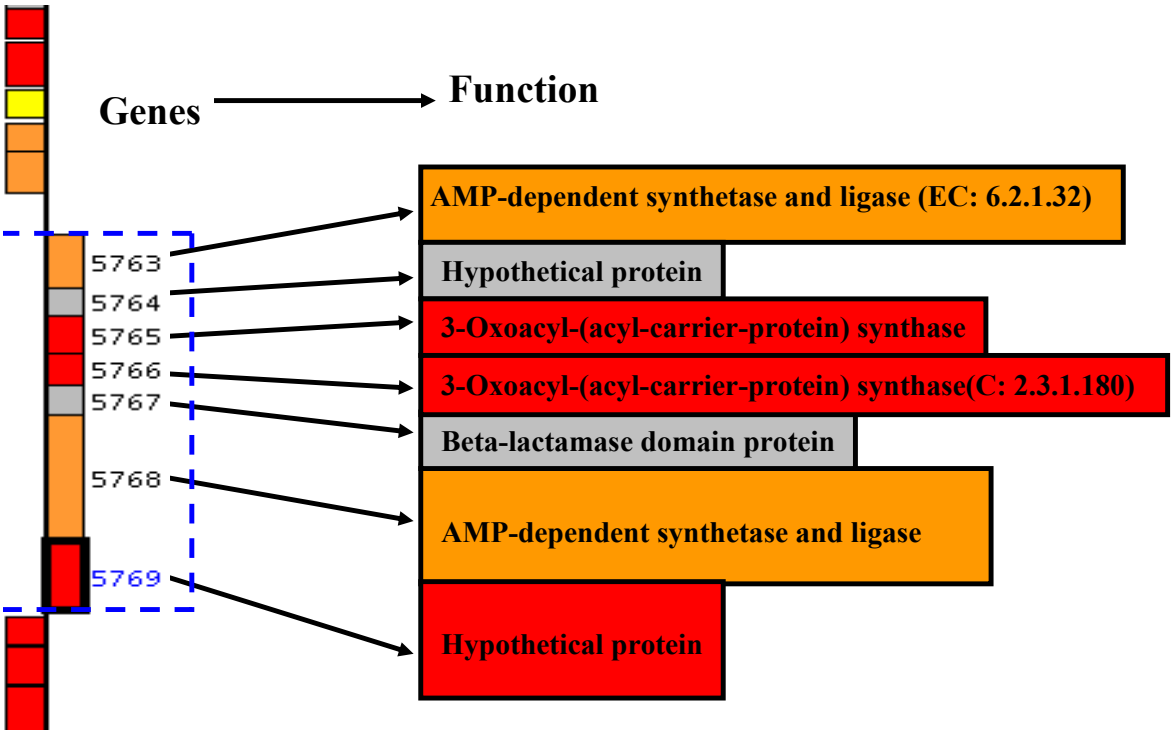

Figure C. 1 The hydroxy-2-alkylquinolines (HQA) cluster of *B. ambifaria* AMMD.

The amino acid and nucleotide sequences of HQA were searched against the *B. ambifaria* AMMD genome using the BLASTN or BLASTP software. The located gene cluster was then marked onto the *B. ambifaria* AMMD genome (Bamb_5763 to Bamb_5769) and navigator tool to match up amino acid or nucleic acid sequence data. The HQA cluster genes and the functional annotation and products of genes are also shown. The color indicated the subcellular localization classes as shown in the key.

Key:

Subcellular Localization Classes
Cytoplasm
Cytoplasmic membrane
Periplasmic
Outer Membrane
Extracellular
Unknown Localization
Unknown Localization (This protein may have multiple localization sites)
No localization assigned and computational localization prediction using PSORTB not performed

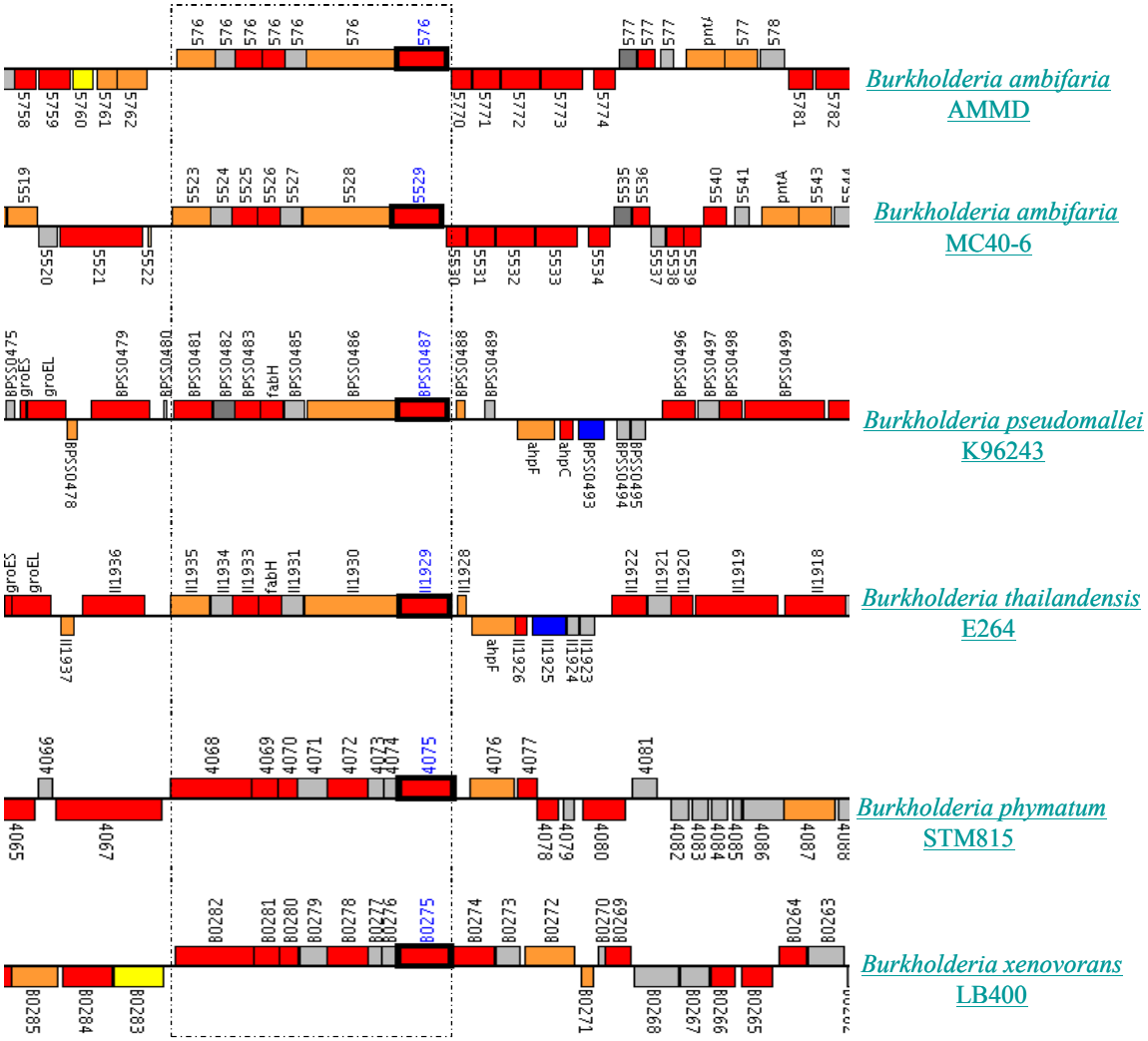

Figure C. 2 Orthologous arrangement of 4 hydroxy-2-alkylquinolines (HQA) biosynthesis genes of *Burkholderia* genomes (Bamb_5763 to Bamb_5769).

HQA genes were identified in 20 *Burkholderia* genomes. The top panel showed *B. ambifaria* AMMD HQA biosynthesis genes, and other five orthologous loci are shown. The color indicated the subcellular localization classes as shown in the key.

Key:

Subcellular Localization Classes
Cytoplasm
Cytoplasmic membrane
Periplasmic
Outer Membrane
Extracellular
Unknown Localization
Unknown Localization (This protein may have multiple localization sites)
No localization assigned and computational localization prediction using PSORTB not performed

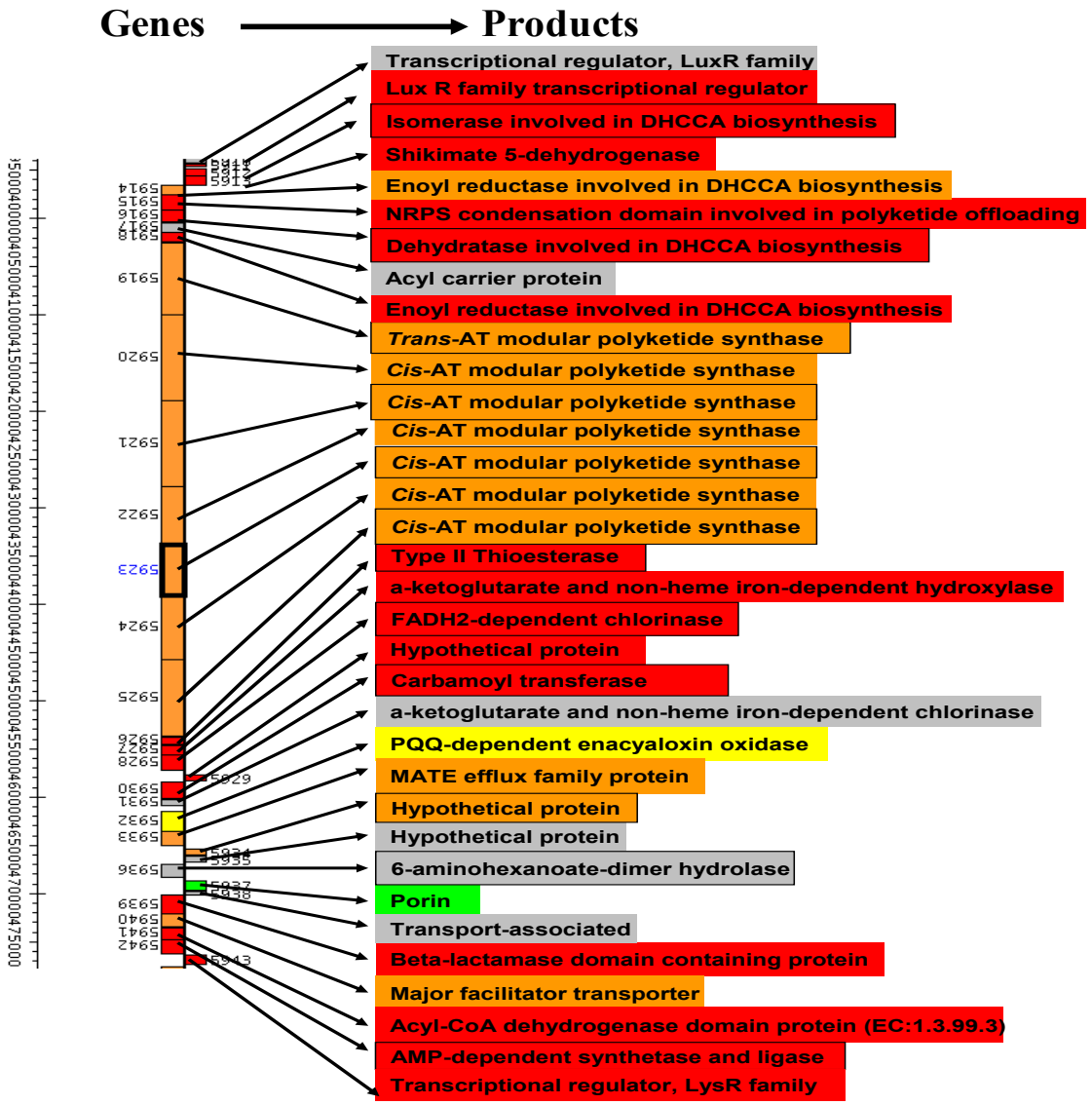

Figure C. 3 The enacyloxin cluster of *B. ambifaria* AMMD.

The amino acid and nucleotide sequences of enacyloxin were searched against the *B. ambifaria* AMMD AMMD genome using the BLASTN or BLASTP software. The location of enacyloxin gene cluster was then marked onto the *B. ambifaria* AMMD genome (Bamb_5910 to Bamb_5943) and navigator tool to match up amino acid or nucleic acid sequence data. Enacyloxin cluster and the functional annotation and products of genes are also shown. The color indicated the subcellular localization classes as shown in the key.

Key:

Subcellular Localization Classes
Cytoplasm
Cytoplasmic membrane
Periplasmic
Outer Membrane
Extracellular
Unknown Localization
Unknown Localization (This protein may have multiple localization sites)
No localization assigned and computational localization prediction using PSORTB not performed

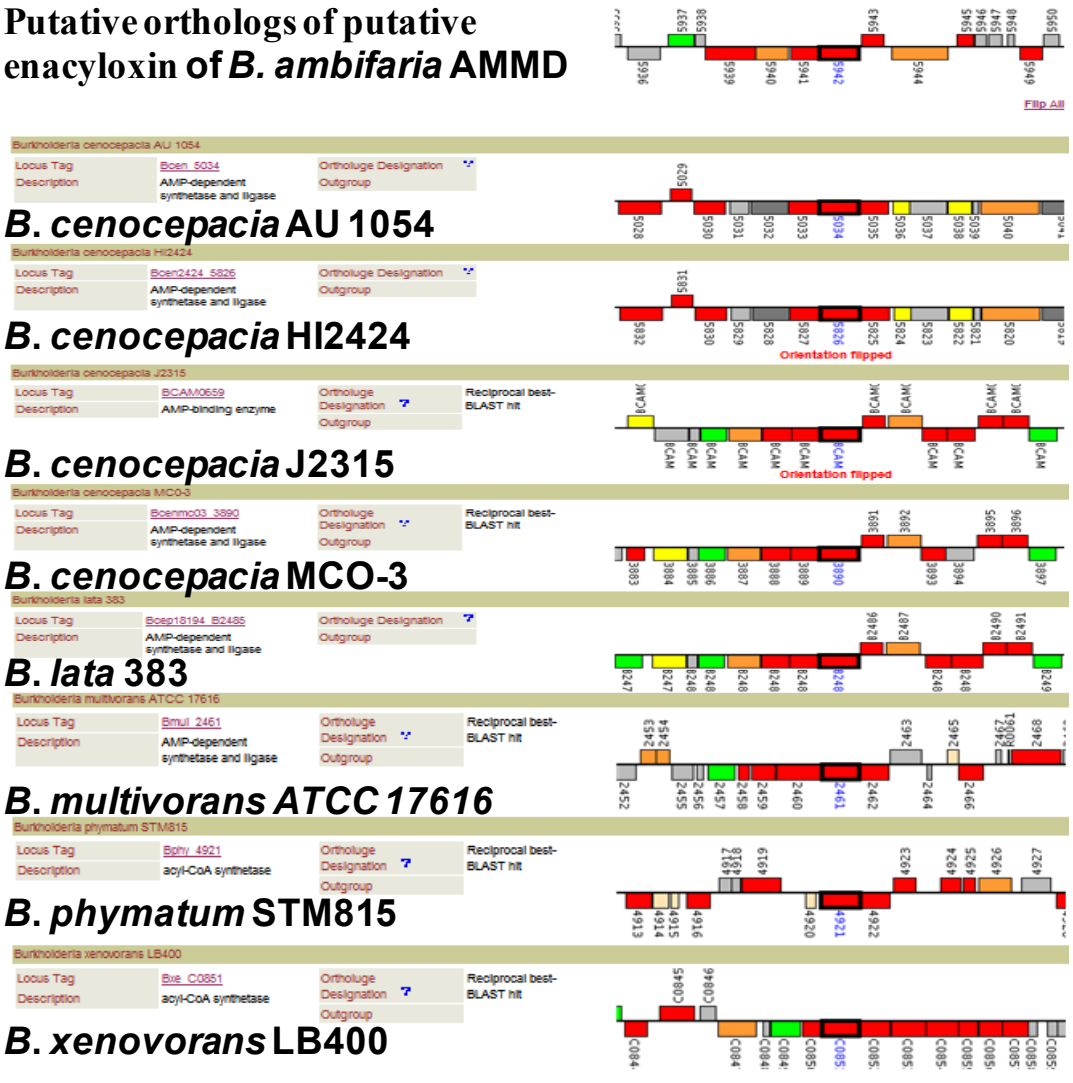

Figure C. 4 Orthologous arrangement of enacyloxin biosynthesis genes of *Burkholderia* genomes.

Enacyloxin genes (Bamb_5910 to Bamb_5943) were identified in 20 *Burkholderia* genomes. The top panel showed *B. ambifaria* AMMD enacyloxin biosynthesis genes, and other eight orthologous loci are shown. The color indicated the subcellular localization classes as shown in the key.

Key:

Subcellular Localization Classes
Cytoplasm
Cytoplasmic membrane
Periplasmic
Outer Membrane
Extracellular
Unknown Localization
Unknown Localization (This protein may have multiple localization sites)
No localization assigned and computational localization prediction using PSORTB not performed

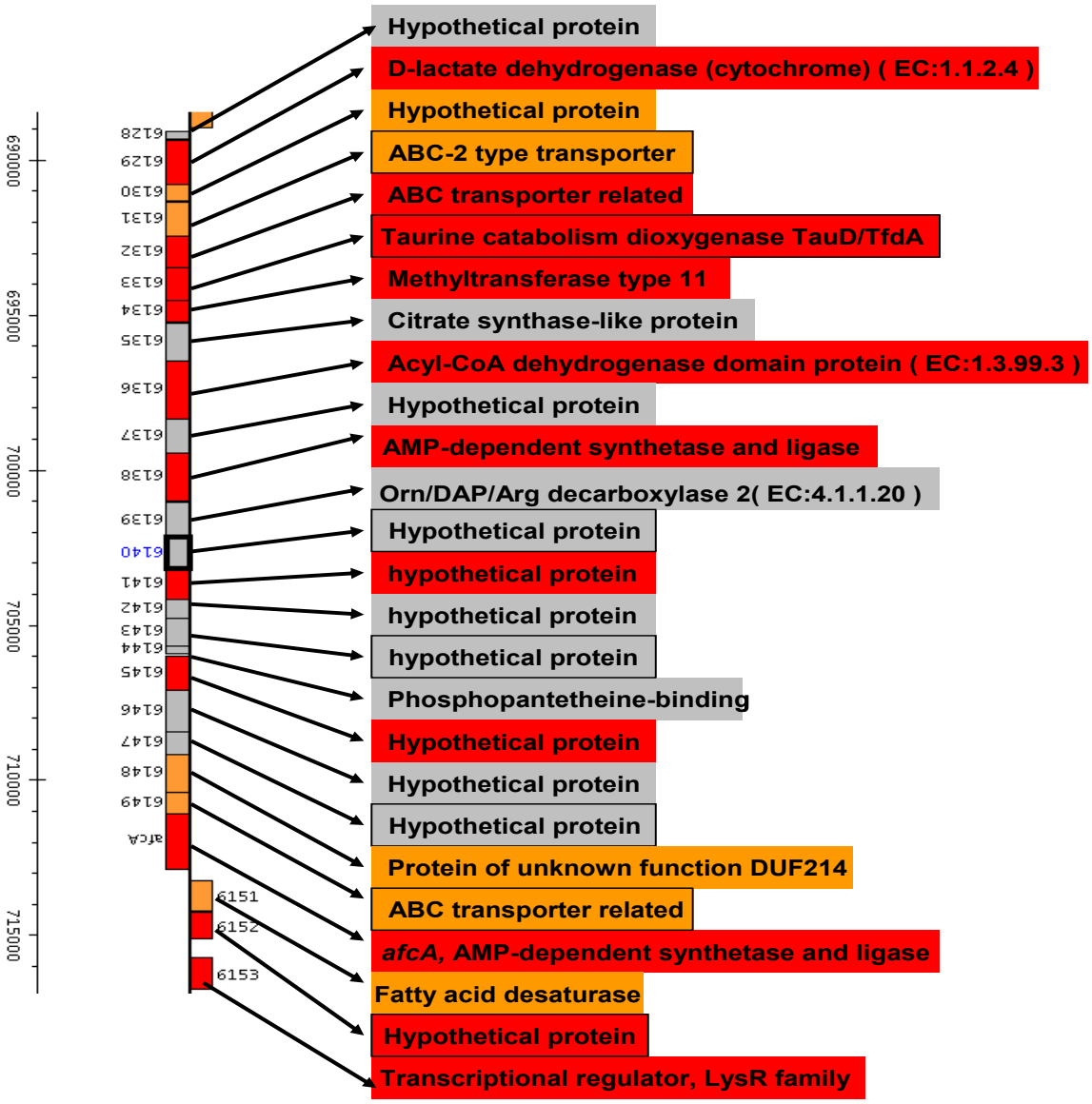

Figure C. 5 The AfcA lipopeptide operon of *B. ambifaria* AMMD.

The amino acid and nucleotide sequences of AfcA were searched against the *B. ambifaria* AMMD genome using the BLASTN or BLASTP software. The location of gene cluster was then marked onto the *B. ambifaria* AMMD genome (Bamb_6128 to Bamb_6153) and navigator tool to match up amino acid or nucleic acid sequence data. The AfcA operon genes, the functional annotation and products of genes are also shown. The color indicated the subcellular localization classes as shown in the key.

Key:

Subcellular Localization Classes
Cytoplasm
Cytoplasmic membrane
Periplasmic
Outer Membrane
Extracellular
Unknown Localization
Unknown Localization (This protein may have multiple localization sites)
No localization assigned and computational localization prediction using PSORTB not performed

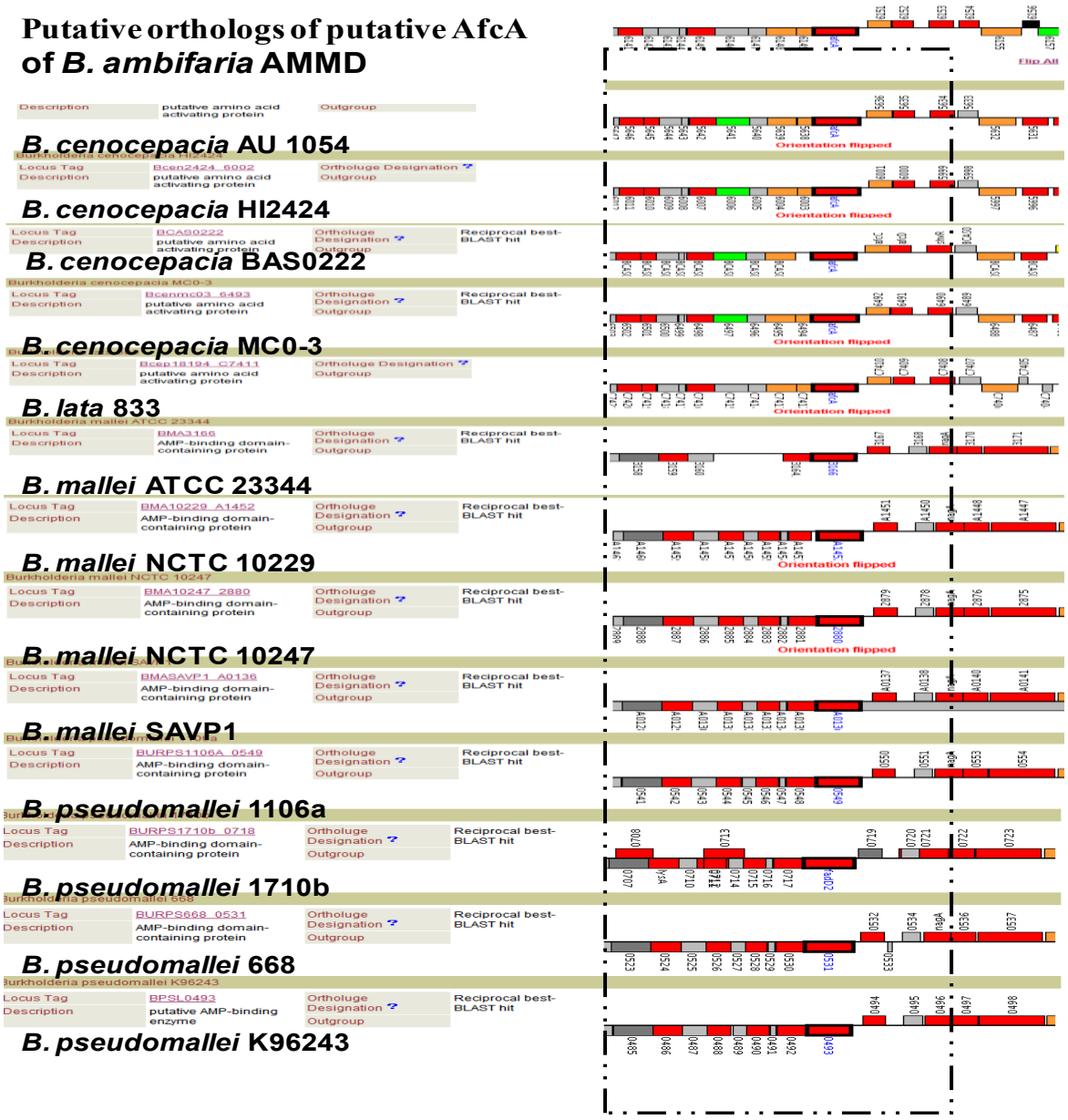

Figure C. 6 Orthologous arrangement of AfcA lipopeptide antibiotic cluster biosynthesis genes of *Burkholderia* genomes.

AfcA genes were identified in 20 *Burkholderia* genomes. The top panel showed *B. ambifaria* AMMD AfcA biosynthesis genes, and other thirteen orthologous loci are shown. The color indicated the subcellular localization classes as shown in the key.

Key:

Subcellular Localization Classes
Cytoplasm
Cytoplasmic membrane
Periplasmic
Outer Membrane
Extracellular
Unknown Localization
Unknown Localization (This protein may have multiple localization sites)
No localization assigned and computational localization prediction using PSORTB not performed

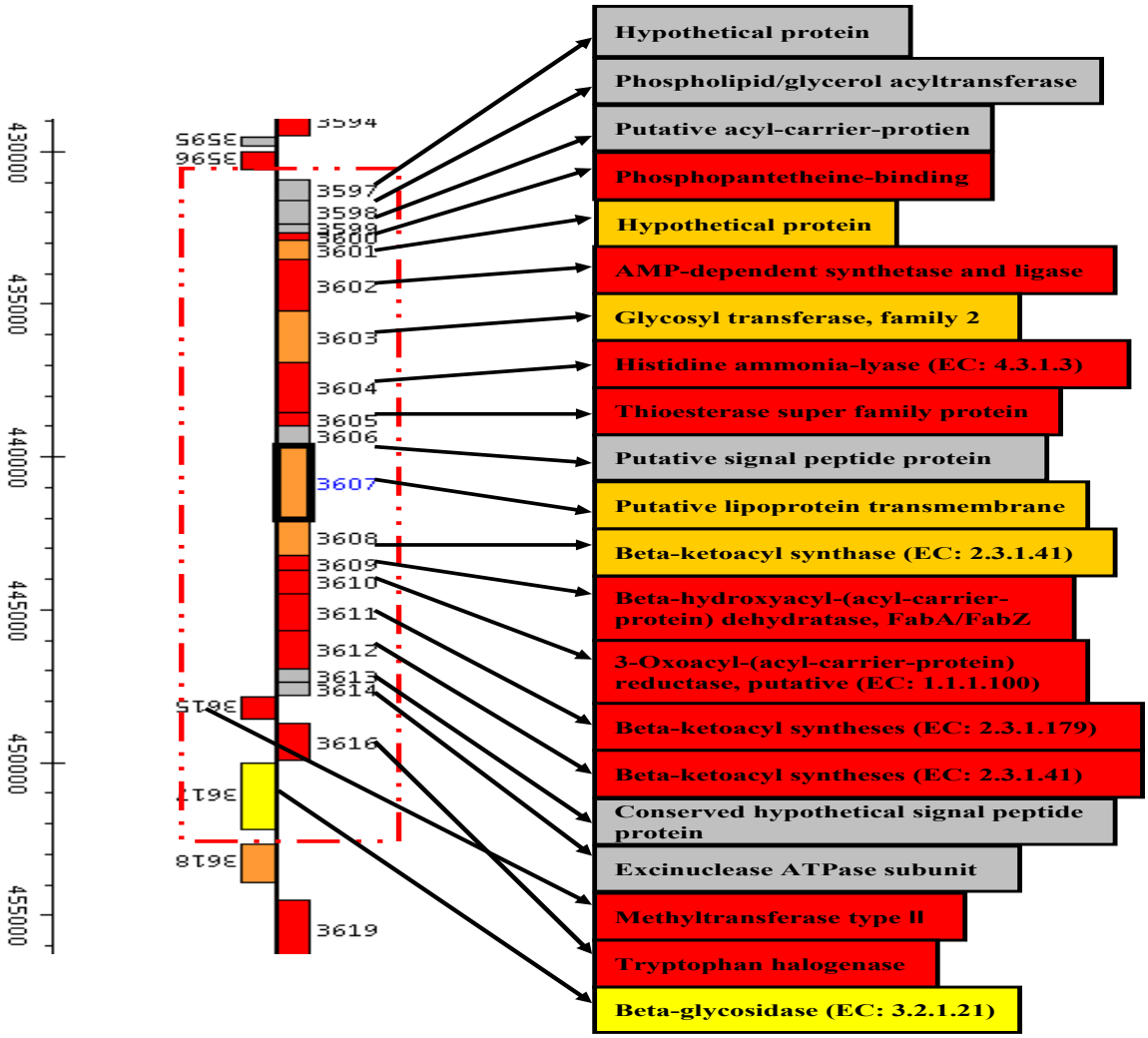

Figure C. 7 The unknown A cluster of *B. ambifaria* AMMD.

The amino acid and nucleotide sequences of unknown A were searched against the *B. ambifaria* AMMD genome using the BLASTN or BLASTP software. The location of unknown A gene cluster was then marked onto the *B. ambifaria* AMMD genome (Bamb_3597 to Bamb_3617) and navigator tool to match up amino acid or nucleic acid sequence data. Unknown A cluster genes, the functional annotation and products of genes are also shown. The color indicated the subcellular localization classes as shown in the key.

Key:

Subcellular Localization Classes
Cytoplasm
Cytoplasmic membrane
Periplasmic
Outer Membrane
Extracellular
Unknown Localization
Unknown Localization (This protein may have multiple localization sites)
No localization assigned and computational localization prediction using PSORTB not performed

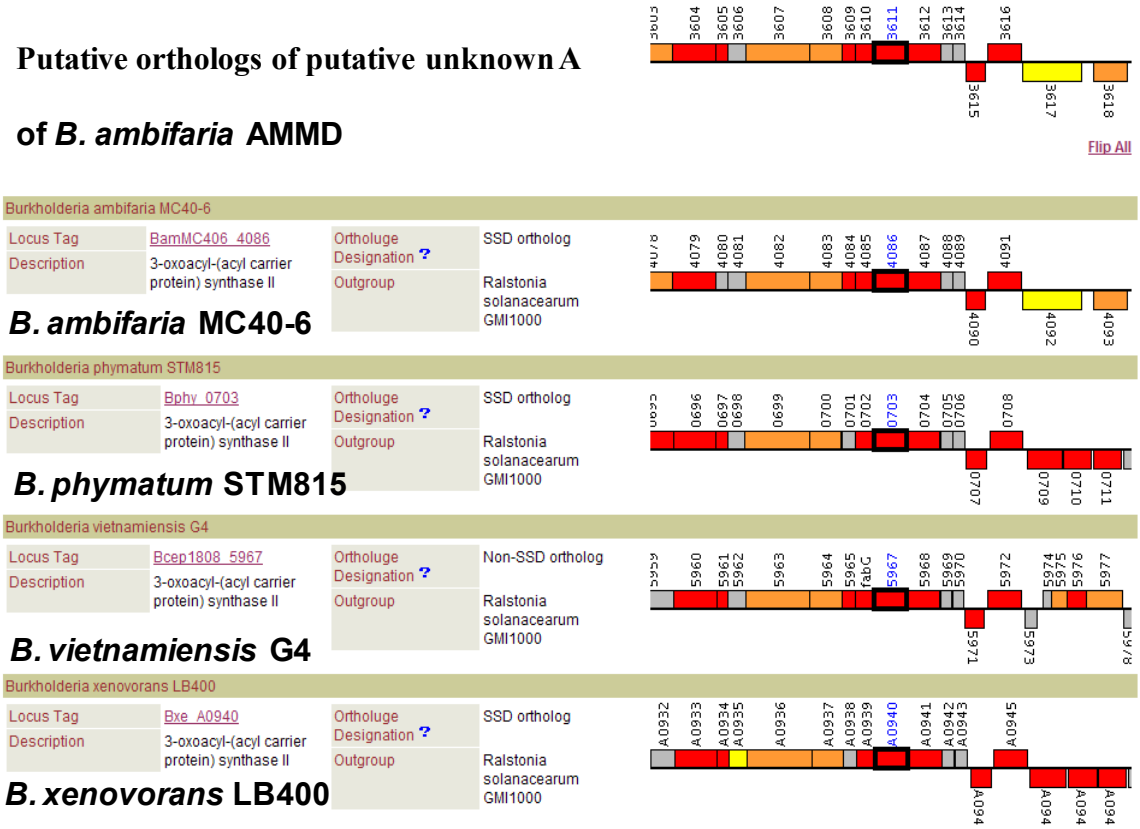

Figure C. 8 Orthologous arrangement of unknown A biosynthesis genes of *Burkholderia* genomes.

Unknown A cluster genes were identified in 20 *Burkholderia* genomes. The top panel showed *B. ambifaria* AMMD unknown A biosynthesis genes, and other four orthologous loci are shown. The color indicated the subcellular localization classes as shown in the key.

Key:

Subcellular Localization Classes
Cytoplasm
Cytoplasmic membrane
Periplasmic
Outer Membrane
Extracellular
Unknown Localization
Unknown Localization (This protein may have multiple localization sites)
No localization assigned and computational localization prediction using PSORTB not performed

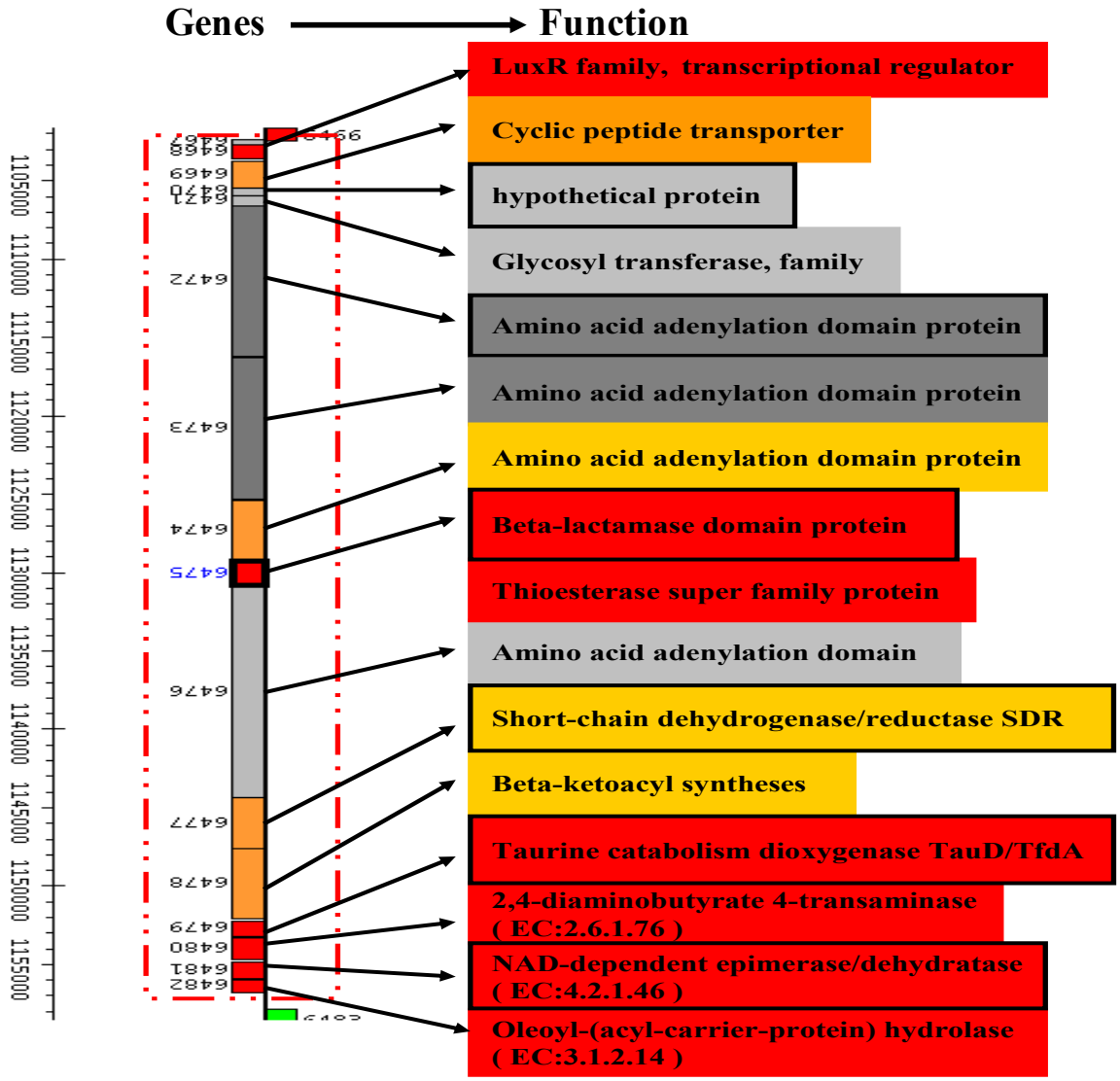

Figure C. 9 The unknown B cluster of *B. ambifaria* AMMD.

The amino acid and nucleotide sequences of unknown B were searched against the *B. ambifaria* AMMD genome using the BLASTN or BLASTP software. The location of unknown B gene cluster was then marked onto the *B. ambifaria* AMMD genome (Bamb_6468 to Bamb_6482) and navigator tool to match up amino acid or nucleic acid sequence data. Unknown B gene cluster, the functional annotation and products of genes are also shown. The color indicated the subcellular localization classes as shown in the key.

Key:

Subcellular Localization Classes
Cytoplasm
Cytoplasmic membrane
Periplasmic
Outer Membrane
Extracellular
Unknown Localization
Unknown Localization (This protein may have multiple localization sites)
No localization assigned and computational localization prediction using PSORTB not performed

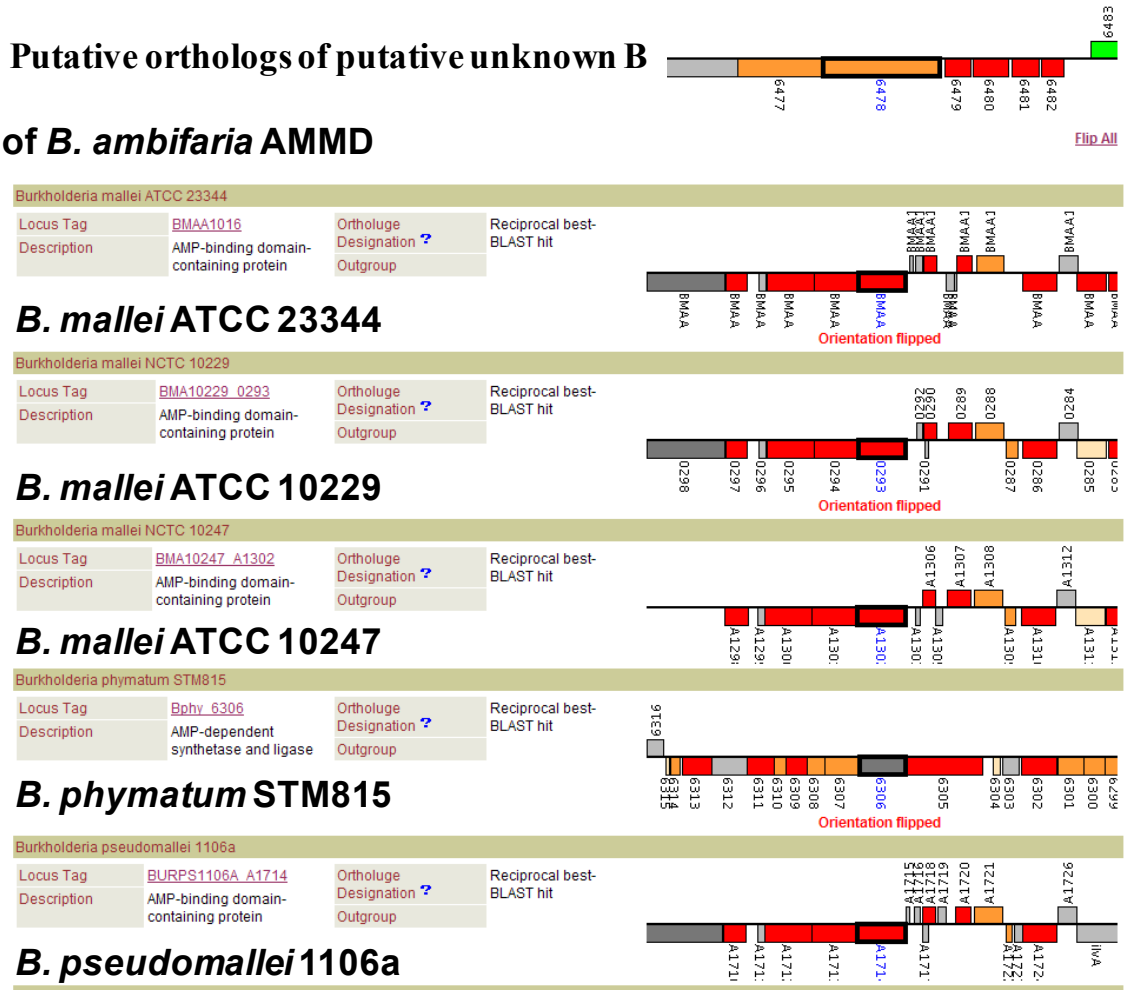

Figure C. 10 Orthologous arrangement of unknown B biosynthesis genes in *Burkholderia* genomes.

Unknown B gene cluster was identified in 20 *Burkholderia* genomes. The top panel showed *B. ambifaria* AMMD unknown B biosynthesis genes, and other five orthologous loci are shown. The color indicated the subcellular localization classes as shown in the key.

Key:

Subcellular Localization Classes
Cytoplasm
Cytoplasmic membrane
Periplasmic
Outer Membrane
Extracellular
Unknown Localization
Unknown Localization (This protein may have multiple localization sites)
No localization assigned and computational localization prediction using PSORTB not performed

APPENDIX D

Enacyloxins Are Products of an Unusual Hybrid Modular Polyketide Synthase Encoded by a Cryptic *Burkholderia ambifaria* Genomic Island

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Summary

Gram-negative *Burkholderia cepacia* complex (Bcc) isolates were screened for antimicrobial activity against cystic fibrosis microbial pathogens, and the ability of *B. ambifaria* to inhibit *B. multivorans* was identified. The activity was mapped to a cluster of cryptic, quorum-sensing-regulated modular polyketide synthase (PKS) genes. Enacyloxin IIa and its stereoisomer designated **iso**-enacyloxin IIa were identified as metabolic products of the gene cluster, which encoded an unusual hybrid modular PKS consisting of multiple proteins with sequence similarity to **cis**-acyltransferase (**cis**-AT) PKSs and a single protein with sequence similarity to **trans**-AT PKSs. The discovery of the potent activity of enacyloxins against drug-resistant bacteria and the gene cluster that directs their production provides an opportunity for engineered biosynthesis of innovative enacyloxin derivatives and highlights the potential of Bcc bacteria as an underexploited resource for antibiotic discovery.

For more details follow this link:

http://www.sciencedirect.com/science/article/pii/S1074552111001190