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CHARACTERIZATION OF THE *OCC* GENE CLUSTER REQUIRED FOR THE PRODUCTION OF ANTIFUNGAL COMPOUND OCCIDIOFUNGION IN *BURKHOLDERIA CONTAMINANS* STRAIN MS14

By

Ganyu Gu

A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Life Sciences
in the Department of Entomology and Plant Pathology

Mississippi State, Mississippi

August 2010

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CHARACTERIZATION OF THE *OCC* GENE CLUSTER REQUIRED FOR THE

PRODUCTION OF ANTIFUNGAL COMPOUND OCCIDIOFUNGION

IN *BURKHOLDERIA CONTAMINANS* STRAIN MS14

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Strain MS14, exhibiting antifungal activity, was classified to belong to *Burkholderia contaminans*. Occidiofungin produced by strain MS14 is an octapeptide dedicated to a broad range of antifungal activities of the bacterium. The 58.2-kb genomic fragment containing 18 open reading frames (ORFs), named occidiofungin (*occ*) gene cluster, is required for occidiofungin production. Putative proteins encoded by five nonribosomal peptide synthetase genes (*occA* – *occE*) of the gene cluster were predicted to contain the catalytic modules responsible for the biosynthesis of occidiofungin. Transcription of all the ORFs identified in the region except *ORF1* and *ORF16* was regulated by both *ambR1* and *ambR2*, the LuxR-type regulatory genes located at the left border of the cluster. The functional *ambR1* gene was essential for transcription of *ambR2*, and constitutive expression of *ambR2* did not restore the phenotype of the mutant MS14GG44(*ambR1*::*nptII*). Sequence analysis revealed that the *occ* gene cluster shared high similarity (99% nucleotide coverage and 91% identity) to an uncharacterized DNA region of *B. ambifaria* strain AMMD. The gene cluster was not found in other

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Burkholderia strains available in GenBank (nucleotide coverage < 24%). Analysis of G+C composition and prediction using "IslandPick" indicate that the *occ* gene cluster has possibly been horizontally transferred between bacteria. In addition, the absence of the gene cluster in clinical strains of *Burkholderia* indicates that occidiofungin is not required for potential human pathogenesis. The findings have provided insights into the development of antifungal medicines and agricultural fungicides based on occidiofungin.

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CHAPTER I

GENERAL INTRODUCTION

Biological control is the use of natural or modified organisms, genes, or gene products, to reduce the effects of undesirable organisms such as plant pathogens and to favor desirable organisms such as crops, and is an important component of integrated pest management (11). It has recently inspired research and development due to the problems associated with hazardous pesticides, such as the negative impact on the environment and the heavy cost. Furthermore, research on novel biological control agents lays the foundation for the development of commercialized products.

Pathogen- Suppressive Soils

Antagonistic microbes present in pathogen-suppressive soils are important resources of genetic materials for developing transgenic crops and biopesticides. Pathogen-suppressive soils were defined as the soils in which a soilborne pathogen does not become established or causes little or no damage to the host plant (11). In agronomic ecosystems, suppressive soils have been described for many soilborne disease systems, such as the diseases caused by *Gaeumannomyces graminis* var. *tritici*, *Pythium* spp., and *Rhizoctonia solani* (41). Bacterial species of the genera *Pseudomonas*, *Bacillus*, *Enterobacter*, and *Streptomyces* as well as species of the fungal genera *Trichoderma* and *Gliocladium* are frequently identified from suppressive soils (29). Therefore, the suppressive soils, in which antagonistic microbes interact with the soilborne pathogens, are important pools to search for useful genetic materials (17). Mechanisms of soil

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suppressiveness to soilborne pathogens are complex, which include antibiotic biosyntheses, resource competitions, and hyperparasitisms (2,12,34,39). One of the best-described examples is the take-all decline soil of wheat fields, in which fluorescent pseudomonads producing antifungal phenazines are present (12). The production of the phenazines was considered to be a key factor to suppress the growth of the take-all pathogen *G. graminis* var. *tritici*. Some strains of *Pseudomonas* identified from the take-all decline soil have been used for biological control of plant diseases (28). However, more studies on diverse disease ecosystems are needed to increase our understanding of molecular activities of these antagonistic microorganisms and to provide more clues to the development of biological-based strategies for disease management (41).

The *Burkholderia* Bacteria

The genus *Burkholderia* was erected by Yabuuchi et al. in 1992 to accommodate a few species of *Pseudomonas* that belongs to the rRNA group II (42). Currently, the genus contains 34 validly described species (9). The bacteria of this genus are attracting considerable attention for its extraordinary versatility as plant pathogens, saprophytes, biocontrol agents, bioremediation agents, and human pathogens (7). Unlike other groups of bacteria, the 16S rDNA sequences of the *Burkholderia* species have significantly high identities (> 98%) and are not sufficiently discretionary to resolve all the species (26). Instead, nucleotide sequence information of the *recA* gene was successfully used to differentiate the *Burkholderia* species from the closely related bacteria and to sort the *Burkholderia* isolates into species (8,27,32). Recently, a polyphasic taxonomic approach using multiple analyses was proposed for identification of species of the species and typing of strains of *Burkholderia* (4).

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A number of *Burkholderia* strains have been reported as plant-growth-promoting bacteria as free-living in rhizosphere, epiphytic, or endophytic microorganisms (7,10,31). Several species of *Burkholderia* were successfully used as biological control agents for managing economically important fungal plant diseases (7). For example, some strains of *Burkholderia* showed significant antagonistic activity against plant pathogens and were commercially registered as biocontrol products, such as "DENY" and "Blue Circle" (http://www.apsnet.org/education/feature/BurkholderiaCepacia/). Some strains of *Burkholderia*, however, were reported to be associated with cystic fibrosis human disease as opportunistic pathogens (26). Unfortunately, taxonomic distinctions have not enabled biological control strains to be clearly distinguished from human pathogenic strains, which has led to a reassessment of the risk of several strains registered by the U.S. Environmental Protection Agency for biological control (31). Therefore, understanding the molecular mechanisms of these bacteria with effectiveness in managing plant diseases might provide important clues to developing biological-based strategies for antifungal applications and to eliminate the potential risk the bacteria may bring.

Antifungal Compounds Produced by the *Burkholderia* Bacteria

Efforts on characterization of chemical structures and genetic information of antifungal compounds have extensively been taken. The antifungal compounds produced by *Burkholderia* bacteria, which have been identified so far, include: cepalycin (1), cepacidine A (23), pyrrolnitrin (18), xylocandin complex (30), CF66I (33), and AFC-BC11 (21).

Cepalycin produced by *B. cepacia* JN106 was identified as a hemolytic antifungal substance, whose chemical composition is unknown (1). It was reported that cepalycins

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could interact with cholesterol in erythrocyte membrane, which might contribute to their hemolytic and antifungal activities.

Cepacidine A produced by *B. cepacia* AF 2001 was identified as a glycopeptide compound comprised of eight amino acids, including one glycine, two serine, one 2,4-diaminobutyric acid, one aspartic acid, one β-hydroxy tyrosine, one β-hydroxy asparagine, one xylose and one 5,7-dihydroxy-3,9-diaminooctadecanoic acid (23) (Fig. 1.1). It was reported that cepacidine A is a mixture of A1 and A2, and the difference between them is that cepacidine A2 includes one asparagine instead of one β-hydroxy asparagine in cepacidine A1.

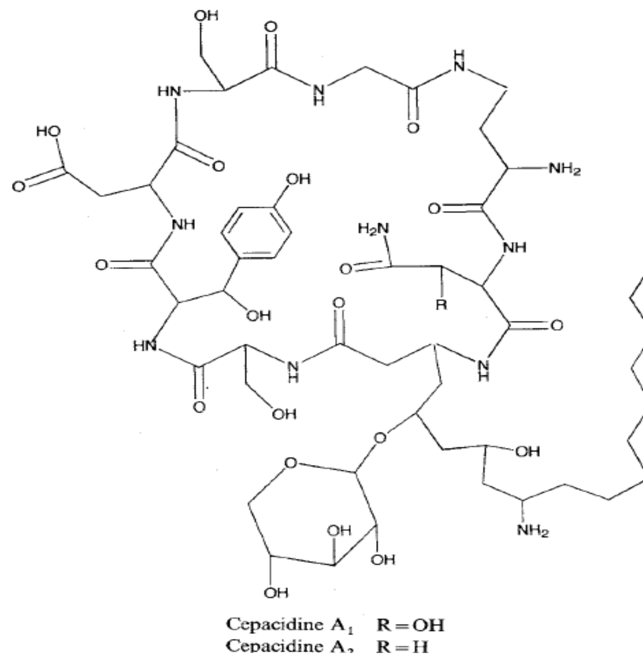

Figure 1.1 Structure of cepacidine A

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Pyrrolnitrin produced by *B. cepacia* NB-1 was identified as $C_{10}H_6C_{l2}N_2O_2$ (18). It can block the electron system, which was relieved by N, N, N, N, tetramethyl-p-phenylenediamine dihydrochloride (TMPD) (Fig. 1.2).

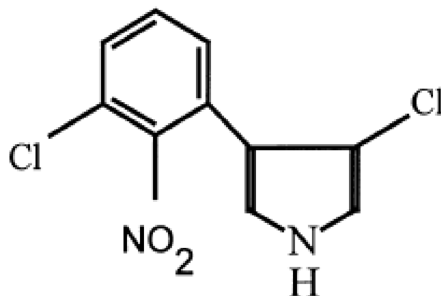

Figure 1.2 Structure of pyrrolnitrin

Xylocandin complex produced by *B. cepacia* ATCC 39277 was identified as a cyclic peptide containing glycine, serine, asparagine (1-3 residues), beta-hydroxytyrosine, and an unusual amino acid with the formula $C_{18}H_{37}N_2O_5$ (5).

CF66I produced by *B. cepacia* CF66 was confirmed to have amide bonds, a-metyl fatty acid, bromine, and some structural units such as CH_2CH_2O (33). It was reported that CF66I was stable to high temperature, proteolytic enzymes, and organic solvents, and it could inhibit the growth of a variety of plant pathogenic fungi and pathogenic yeast, whereas bacterial cells are unaffected.

AFC-BC11 produced by strain *B. cepacia* BC11 was predicted to be a nonribosomally synthesized lipopeptide (21). One of the genes required for biosynthesis of AFC-BC11 was partially characterized (21).

Biosyntheses of Antifungal Compounds in *Burkholderia*

Among these antifungal compounds, only cepacidine A, xylocandin complex and AFC-BC11 were characterized or predicted to be oligopeptides. Only the genes required

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for biosynthesis of AFC-BC11 and pyrrolnitrin were partially characterized; while the genes for production of the rest of substances remain to be characterized. Few genetic elements that are responsible for antimicrobial activities of the *Burkholderia* strains have been characterized. A major limitation for the genetic analysis of the biological functions of the bacteria is that tools for genetic manipulations and analysis are much less well developed because most strains of *Burkholderia* possess inherent resistance to many antibiotics and lower frequencies of the introduction of foreign plasmids by both electroporation and conjugation (20). In addition, the genomes of the species of *Burkholderia* are much larger and more complicated than those of pseudomonds because each genome of the bacteria is composed of multiple replicons (23).

N-acyl homoserine lactones (AHLs) synthesized by AHL synthases in *B. ambifaria* BC-F were identified as signal chemicals interacting with transcriptional regulators of the LuxRI family (43). It was reported that the AHL-deficient mutants of *B. ambifaria* BC-F at AHL synthase gene (*bafI*) and AHL-binding transcriptional activator gene (*bafR*) positions had decreased antifungal activity.

Nonribosomal peptides, as a class of peptide secondary metabolites, are synthesized by NRPS, which, unlike the ribosomes, are independent of messenger RNA (38). Nonribosomal peptide synthetase typically is a large multimodular enzyme involved in oligopeptide biosynthesis. NRPSs are grouped by active sites termed modules, in which each module is required for catalyzing one single cycle of product length elongation (Fig. 1.3). Nonribosomal machinery for peptide synthesis is usually composed of more than one NRPS modules, which form a multienzyme complex as an assembly line to catalyze stepwise peptide condensation. The order and number of the modules of an NRPS protein are, in many cases, collinear to the amino acid sequence of

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the corresponding peptide moiety of the final molecule ('collinearity rule') (16) . Each module of the NRPSs can be further subdivided into domains, each of which exhibits a single enzymic activity. The adenylation (A) domain is responsible for amino acid recognition and adenylation at the expense of ATP. The thiolation (T) or peptidyl carrier protein domain is the attachment site of the 49-phosphopantetheine cofactor and serves as a carrier of thioesterified amino acid intermediates. The condensation (C) domain catalyses peptide bond formation between two consecutive amino acids. Modifying domains such as the epimerization (E) domain catalyse the conversion of L-amino acids to their D-isomers. Finally, cyclization and release of the peptide product are catalysed by the C-terminal thioesterase (Te) domain. Thioesterase domains often occur integrated in or associated with peptide synthetases which are involved in the non-ribosomal synthesis of peptide antibiotics (39). Thioesterases are required for the addition of the last amino acid to the peptide antibiotic, thereby forming a cyclic antibiotic (6). Many pharmaceutical antimicrobial agents are biosynthesized via NRPS systems, such as penicillin precursor and vancomycin (40). Common to NRPS assembly lines are the incorporation of nonproteinogenic amino acids, such as D-amino acids and carboxy acids (38). Sequence analyses of catalytic modules of NRPSs are frequently used for product prediction (35).

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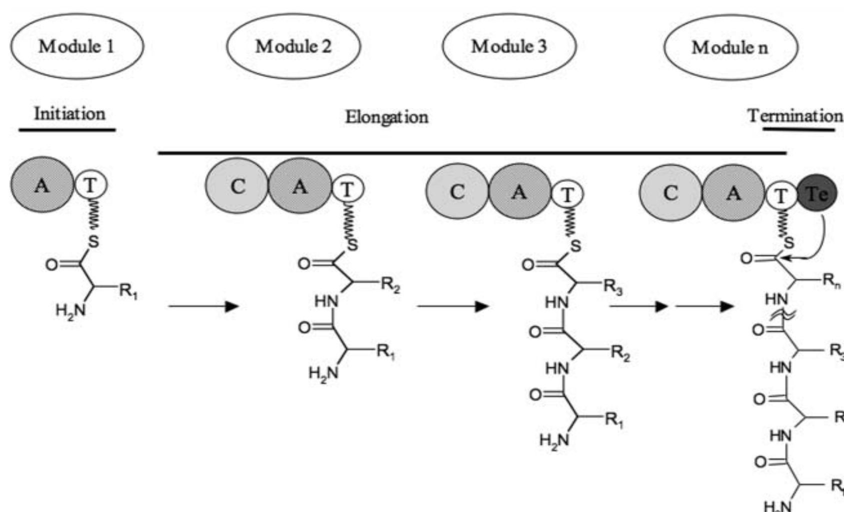

Figure 1.3 Basic steps during non-ribosomal peptide synthetase

Polyketide synthetases (PKS) are a family of enzymes or enzyme complexes that produce polyketides, a large class of biologically active molecules, in bacteria, fungi, plants, and a few animals lineages (22). The biosyntheses of PK share striking architectural and organizational similarities with NRP biosynthesis with the building block of propionyl-CoA or methylmalonyl-CoA instead of amino acid. Several classes of PKS genes are commonly recognized (22). Type I PKS are large, multidomain enzymes carrying a series of functional sites for stepwise polyketide synthesis. Type II PKS are composed of three or more enzymes, which act in an iterative manner during synthesis. A core of three enzymes referred to as the minimal PKS is shared by all pathways: the Acyl transferase (AT) domain, the Acyl carrier protein (ACP) domain, and the Ketosynthase (KS) domain (Fig. 1.4). Polyketides are structurally diverse secondary metabolites that have already found widespread application as pharmaceuticals, such as erythromycin (13).

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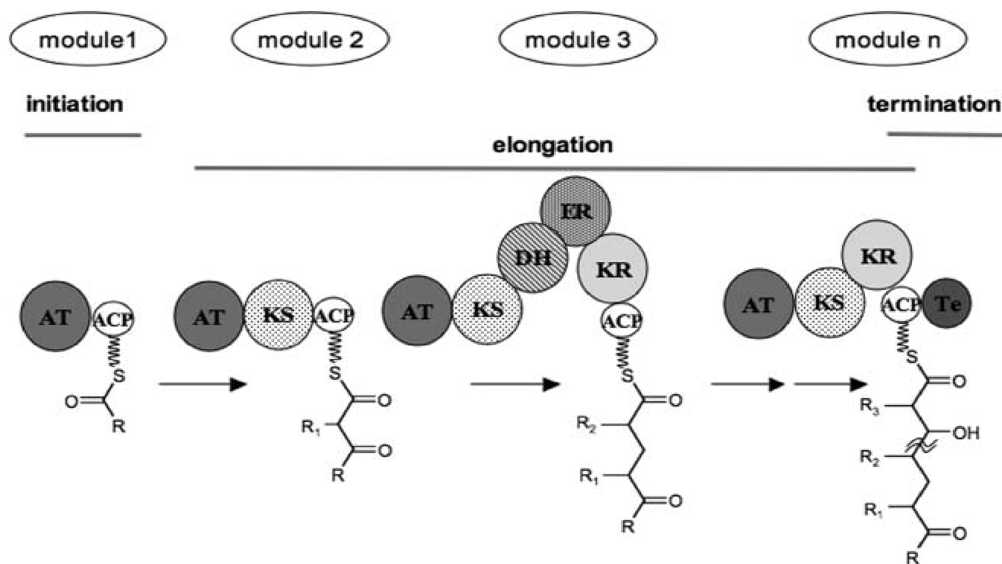

Figure 1.4 Basic steps during polyketide synthetase

The modules of NRPS and PKS can be integrated to produce the hybrid NRPS-PKS products (3), such as bleomycin produced by the *Streptomyces verticillus* and gramacidin S produced by *Bacillus brevis*.

LuxR-type regulators, which contains DNA-binding, helix-turn-helix (HTH) domains at their C termini, are conserved and common proteins in bacteria (14,19,25). These LuxR type regulators control a wide variety of biological functions, including production of secondary metabolites, such as NRPS-PKS products. Based on their functional mechanisms, these LuxR type proteins are grouped into two major subfamilies: response regulators and autoinducer-binding regulators (19).

Significance of the Study

In this research, a few isolates with significant antifungal activity were isolated from agricultural soils of Mississippi. Evidence from morphology, biochemistry and DNA sequence analysis revealed that one of the isolates, named as MS14, was characterized to belong to *B. contaminans*. Strain MS14 is predicted to produce a novel

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oligopeptide antifungal compound via a hybrid nonribosomal peptide synthetase (NRPS) and polyketide synthetase (PKS) system.

This study reveals that the *occ* gene cluster is responsible for the production of antifungal compound of strain MS14, which has provided a firm base for characterizing the biosynthesis and chemical composition of the antifungal compound produced by strain MS14. This work has also provided important clues for development of pharmaceutical medicines and agricultural fungicides.

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CHAPTER II

BIOSYNTHESIS OF AN ANTIFUNGAL OLIGOPEPTIDE IN *BURKHOLDERIA CONTAMINANS* STRAIN MS14

Introduction

The *Burkholderia cepacia* complex (Bcc) was composed of nine validly named species of *Burkholderia*, which are genetically different but phenotypically similar bacteria (4). Recently, the descriptions of an additional eight novel species have increased the number of Bcc species to seventeen (24) including *B. contaminans* (23). Nucleotide sequences of the *recA* genes have been used to differentiate the *Burkholderia* species and all members of the *B. cepacia* complex (16).

A striking feature of some strains of *Burkholderia* is production of various antifungal compounds, which can be used for fungal disease management (4). However, their use for fungal disease management was reconsidered, since they were shown to be opportunistic pathogens associated with the human disease cystic fibrosis (15). Understanding the molecular mechanisms of antifungal activities of the *Burkholderia* strains will provide important clues for the development of biological-based fungicides, while eliminating potential health risks. However, few genetic elements responsible for antimicrobial activities of *Burkholderia* have been characterized, particularly due to genetic analysis limitations of the biological functions of the bacteria. Some strains have an inherent resistance to many antibiotics and the development of genetic analysis tools had been lacking (10). The development of the Tn*5* transposition complexes (8), as well

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as the *Burkholderia* expression vectors (12) has enabled the characterization of genes of interest more efficiently.

Strain MS14 showed a broad range of antifungal activities to plant and human fungal pathogens (14). In this study, the bacterium was classified to belong to *B. contaminans*, and a 22.7-kb genetic locus was demonstrated to be required for the antifungal oligopeptide biosynthesis.

Materials and Methods

Culture condition and media

Nutrient broth-yeast extract (NBY) (9) was used to culture strains of *Burkholderia*. Potato dextrose agar (PDA) and potato dextrose broth (PDB, Difco Laboratories Inc., Detroit MI) were used for plate bioassays of *Burkholderia* strains for antifungal activity and for isolation of antifungal compounds, respectively. When required, antibiotics were added at the following concentrations: trimethoprim (50 μg ml^{-1}) and kanamycin (100 μg ml^{-1} for *E. coli* and 300 μg ml^{-1} for the MS14 mutants).

Assays of antifungal activities

Antifungal activities of strain MS14 and its mutants were evaluated using the PDA plate bioassays described previously (22). *Geotrichum candidum* F-260 (9) was used as a indicator fungus for the assays. Fractions from the reverse phase-high performance liquid chromatography (RP-HPLC) run were dried in a speedvac at room temperature and resuspended in an equal volume of water. The bioactivity of the fractions was measured routinely using a fungicide assay method (5).

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Cloning and sequence analysis of the 16S rRNA and recA genes

Extraction of bacterial genomic DNA was performed using the cetyl trimethyl ammonium bromide protocol (3). Primers Bu16SF and Bu16SR (Table 2.1) were used to amplify the 16S rRNA gene of strain MS14 using a routine PCR procedure (20). The same PCR procedure was conducted for the *recA* PCR amplification with primers BCR1 and BCR2 as described previously (16) except using Vent DNA polymerase (New England Biolabs, Beverly, MD). After adenylation, the PCR products were ligated into pGEM T-Easy vector as recommended by the manufacturer (Promega Corp., Madison, WI). Sequencing reactions were run on a CEQ 8000 genetic analysis system (Beckman Coulter, Inc., Fullerton, CA). The Lasergene software package (DNASTAR, Inc., Madison, WS) was used for sequence analysis.

Mutagenesis analysis

To characterize the genes dedicated to antifungal activity, strain MS14 was randomly mutated using an EZ-Tn5 <R6Kγ*ori*/KAN-2>Tnp Transposome kit as recommended by the manufacturer (Epicentre Biotechnologies, Madison, WI). Insertion of the EZ::TN transposon conferred resistance to kanamycin at 300 μg ml^{-1} supplemented on NBY plates, enabling selection of mutant candidates. The mutants that were defective or reduced significantly in antifungal activity against *G. candidum* were further streaked on NBY plates for further analysis. To confirm that the resulting mutants were derivatives of strain MS14, the *recA* genes were cloned and sequenced as described above. The single insertion of the transposon into the genome of each resulting mutants was verified by Southern blotting analysis using the *nptII* gene fragment (13). The DNA fragments targeted by the transposon in each of the mutants were cloned using a plasmid

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rescue procedure as recommended by the manufacturer of the EZ-Tn5 kit. Sequencing reactions were performed using the primers supplied in the kit.

Construction of a genomic library of strain MS14, library screening, and DNA sequencing

To characterize the intact genes of interest, a genomic library of strain MS14 was constructed using the CopyControl HTP Fosmid library production kit as recommended by the manufacturer (Epicentre Biotechnologies). To identify the clones harboring the gene fragment of interest, the genomic library was screened using a 0.4-kb PCR product, which carries the partial gene transposon-targeted in mutant MS14MT15, following a routine colony hybridization protocol (20). The 0.4-kb PCR product was amplified from 100 ng plasmid pSL615 (see description in Results and Discussion) using a GoTaq PCR Core kit (Promega) with primers R6KAN-2RP-1 and KAN-2FP-1 provided in the EZ::TN transposon kit. The DNA insert of fosmid 5F12 identified from the library was sequenced by using a EZ-Tn5-based sequencing approach (21). At least triple coverage of sequencing reactions was achieved, and a consensus sequence was generated with the SeqMan program in the Lasergene software package, and the resulting sequence was deposited into the GenBank database with accession number: EU938698. The program BLAST (2) was used to identify nucleic acid and protein homologies. InterProScan program was used for prediction of functional domains and signatures of proteins (18). NRPSpredictor was used for specificity prediction of adenylation domains in nonribosomal peptide synthetases (NRPSs) (19).

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Complementation of mutant MS14MT15

To obtain the intact wild type gene *ambR2*, which was disrupted in mutant MS14MT15, a 1.9-kb *Bam*HI fragment of the fosmid 5F12 was subcloned into pBlueScript SK (+) generating plasmid pGG1. The presence of the intact *ambR2* gene in the 1.9-kb DNA fragment was verified by sequencing. The 1.9-kb *Bam*HI fragment harboring the intact *ambR2* gene was inserted into the *Burkholderia* gene expression vector pMLS7 (12), to generate plasmid pGG2. Plasmid pGG2 were electroporated into cells of mutant MS14MT15. The empty vector was used as a control. Colonies acquiring the trimethoprim resistance were confirmed to contain the plasmid construct pGG2 by sequencing. Complementation experiments were conducted using the plate assays to evaluate antifungal activity against *G. candidum*.

RNA extraction and quantitative real-time PCR

Real time PCR analysis of the ORF5 expression indicated that the maximum level of RNA was present in MS14 growing in 100 mL of PDB for 72 hr after inoculation at 28°C without shaking. Consequently, RNAs were extracted under this condition for gene expression analyses. Total RNA of bacterial cells was isolated using an RNeasy Protect Bacteria kit (Qiagen, Valencia, CA) as recommended by the manufacturer. The One-Step QuantiTect SYBR green RT-PCR kit (Qiagen) was used with primers (Table 2.1) for each gene. The C_T values of the ORFs used to determine transcript levels were normalized by housekeeping gene *recA* as follows: $\Delta C_T = C_{T(\text{tested})} - C_{T(recA)}$. The relative quantification (RQ) values were calculated by the formula $RQ = 2^{[\Delta C_{T(\text{mutant})} - \Delta C_{T(\text{wild type})}]}$. As described previously (6), if transcript levels of the wild type and the mutant have no differences, RQ = 1 and log RQ = 0. Three replicates of RT PCR analysis for each reaction were performed independently. Statistical significant differences were

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determined for RQ values by analysis of variance ($P < 0.05$) followed by the Bonferroni and Dunnet post hoc multiple comparisons (ANOVA, SAS Institute, Inc., NC, USA).

Isolation of antifungal compound

The wild type strain MS14 (200 µl at 4×10^7 CFU ml^{-1}) was inoculated into 100 ml PDB and incubated at 28°C for 4 days without shaking. The mutant MS14MT18 was used as a control, since it lacks antifungal activity. Ammonium sulfate (AS) precipitations (50% w/v) were done using 30 ml of the cell free culture liquor on ice for 2 hours. Following the AS precipitation, supernatants were centrifuged at 10,000 × *g* for 20 minutes. The pellets were resuspended in 1 ml of 35% acetonitrile (ACN):water (v/v) and placed in a 1.5 ml microcentrifuge tube. RP-HPLC was done using a 4.6 × 250 mm C18 column (Grace-Vydac, catalog 201TP54) on a Bio-Rad BioLogic F10 Duo Flow with Quad Tec UV-Vis Detector system. Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry – Time of Flight (MALDI-TOF, ABI 4700 Proteomics Analyzer) was used to determine the mass of peaks that weren't in common to MS14MT18 RP-HPLC fractions. Amino acid analysis was performed at the Molecular Structure Facility, UC Davis. The bioactivity of these fractions was determined using the antifungal bioassay described above.

Results and discussion

Classification of strain MS14

A nearly full length of the 16S rRNA gene sequence was determined for strain MS14 (GenBank accession no. EU938697), and shared the highest identities (98.0-99.7%) to those of the *Burkholderia* species. A 1,003 bp PCR fragment carrying the *recA*

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gene of strain MS14 was obtained and sequenced (GenBank accession no. EU938696). Sequence analysis revealed that the nucleotide sequence of the *recA* gene of strain MS14 was identical to those of both *B. contaminans* (*vanimaris*) R-9896 (GenBank accession no. AM905036) and *B. contaminans* (*cepacia*) R-9929 (Elke Vanlaere, personal communication; GenBank accession no. AF456020) (23). The results suggest that strain MS14 is a member of the species *B. contaminans.* Strains characterized in the species were primarily isolated from cystic fibrosis patients, as well as sheep milk (23). Strain MS14 was isolated from plant rhizosphere, which has expanded the distribution of *B. contaminans*.

Identification of three genes involved in production of the antifungal product

From three transposition events using the EZ-Tn5 transposon system approximately 8,600 kanamycin resistant colonies were obtained and then tested for antifungal activity against *G. candidum*. From the preliminary screening, it was found that the antifungal activity of three mutants of strain MS14 were reduced (MS14MT15) or eliminated (MS14MT13 and MS14MT18). As compared with the wild type MS14, antifungal assays revealed that mutants MS14MT13 and MS14MT18 were defective in antifungal activity against the indicator fungus *G. candidum*, and the antifungal activity of mutant MS14MT15 was reduced by approximately 80% (Fig. 2.1I). The three mutants also showed the same patterns of antifungal activity against *R. solani* (data not shown).

Three plasmids pSL613, pSL615 and pSL618 were obtained from the genomes of mutants MS14MT13, MS14MT15 and MS14MT18, respectively, using the plasmid rescue method. Plasmids pSL613, pSL615 and pSL618 carry 0.3 kb, 0.5 kb and 2 kb genomic DNA inserts, respectively. The genes targeted by the transposon were cloned

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and partially sequenced. BLAST analyses revealed that the disrupted genes of the mutants MS14MT13, MS14MT15, and MS14MT18 shared high identities to bamb_6472, bamb_6468, and bamb_6473, respectively, in an uncharacterized DNA region of *B. ambifaria* AMMD (GenBank accession no. NC_008392).

Sequence analysis of the gene region responsible for production of the antifungal product

Assembling sequencing reads generated a 22.7-kb DNA sequence contig from the fosmid 5F12. The nucleotide sequence including putative proteins was deposited in GenBank (Accession number: EU938698). Sequence analysis revealed the presence of six potential ORFs in the region (Fig. 2.2). Genetic organization and transcriptional orientations of the predicted ORFs in the DNA region are the same as those of strain AMMD. The six proteins predicted in the DNA region have an averaged identity of 86.7% to those of strain AMMD (Table 2.2).

The *ambR2* gene was predicted to start with an ATG translation initiation codon that is preceded by a putative Shine-Dalgarno sequence (AGGA), and it codes for a 296-residue protein. Database search showed that the full length AmbR2 protein had strong amino acid identity (77%) to the protein Bamb_6468, a putative LuxR regulator of strain AMMD. Alignment analysis of the DNA sequence previously obtained with the plasmid rescue method revealed that the transposon in the mutant MS14MT15 genome was inserted within the *ambR2* gene between nucleotides 254 and 255 relative to its start codon.

Putative protein sequences of the ORFs 5 and 6 are 3165 and 3022 amino acids in length, respectively. Both proteins shared significant identities to two NRPSs produced by a gene cluster in the genome of strain AMMD. ORF5 was predicted to have two

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NRPS catalytic modules for asparagine and serine, followed by a thioesterase domain at the C terminus (a.a. 2,892-3,159; E score: 2.3e-23). ORF6 was predicted to have two catalytic modules of NRPS, however, no amino acid matches from the database were found. These results suggest that the two NRPS genes contribute to catalyzation of four amino acid residues of an unknown oligopeptide. Sequence analysis revealed that the transposons in the genomes of mutants MS14MT13 and MS14MT18 were located between nucleotides 7,336-7,337 of ORF5 and 4,648-4,649 of ORF6, respectively, relative to their translational start codons. These mutations resulted in a complete loss of antifungal activity.

NRPS genes are widely distributed in the bacterial genomes and frequently clustered together with transcriptional regulator genes and transporter genes (6,13). The NRPS contribute to biosynthesis of oligopeptides or oligopeptide-based metabolites including various antibiotics (17). The findings of this study suggest that the antifungal compound of MS14 is an oligopeptide synthesized via a nonribosomal peptide synthetase mechanism.

Characterization of AmbR2's role in the production of the antifungal product

The *ambR2* gene targeted in the transposon mutant MS14MT15 was further characterized to determine the gene's direct role in antifungal production. Introduction of plasmid pGG2, carrying the wild type *ambR2* gene, into the mutant complemented the mutation *in trans*. Mutant MS14MT15 harboring plasmid pGG2 was as inhibitory to *G. candidum* as the wild type strain MS14 (12.0 ± 1.0 mm in radius) (Fig. 2.1II). In contrast, mutant MS14MT15 carrying the empty vector pMLS7 had the same antifungal activity as the mutant (2.7 ± 1.2 mm in radius). Furthermore, the presence of the empty

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vector pMLS7 had no effect on antifungal activity of the wild type strain MS14 (data not shown). These results show that *ambR2* plays a direct role in production of antifungal activity of MS14.

To test whether a mutation in *ambR2* affected expression of other ORFs in the region, quantitative real-time PCR analyses were performed with RNAs isolated from the wild type strain MS14 and MS14MT15. Significant decreases ($P < 0.05$) of expression levels of ORFs 2-6 in MS14MT15 were observed as compared with those in the wild type strain MS14. Transcript levels of the ORFs in MS14MT15 (as compared with the wild type MS14) were reduced 11.5 folds on average (Fig. 2.3). These data reveal that *ambR2* gene product acts to promote, either directly or indirectly, transcription of ORFs 2-6.

Isolation of antifungal compound produced by strain MS14

The mutant MS14MT18 was used as a control for the isolation of the antifungal compound by RP-HPLC, since it lacks antifungal activity to the indicator fungus *G. candidum*. As seen in Fig. 2.4, mutant MS14MT18 (gray) lacked peaks that were present in the wild type strain MS14 (black). Further analysis indicated that a doublet peak in the RP-HPLC chromatogram exhibited strong antifungal activity to the indicator fungus (data not shown). The antifungal compound eluted from the column at 37% water:ACN 0.1% TFA (v/v). The molecular weights of the purified compound were determined to be 1200.6 Da and 1216.6 Da. Presumably this mass difference of 16 Da is attributed to an oxidized variant of the antifungal compound. An additional peak found in the AS precipitant of MS14, not MS14MT18, was isolated, and determined to have no antifungal activity and a mass of 1072.8 and 1088.8 Da. Possibly this peak could be a variant of the

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antifungal compound that has not undergone complete processing, since it also appears to vary by 16 Da. Amino acid analysis of the purified bioactive peak revealed that the antifungal compound consists of asparagine or aspartic acid, glycine, lysine, and serine residues. Composition of serine and asparagine is consistent with the catalytic modules of the NRPS predicted in ORF5. These data further suggest the antifungal compound is an oligopeptide-based product.

To date, only a few (full or partial) chemical compositions of antifungal compounds produced by *Burkholderia* have been reported, such as cepalycin (1), cepacidine A (11), pyrrolnitrin (7), and AFC-BC11 (10). In addition, the genes required for these products were partially (AFC-BC11) or fully (pyrrolnitrin) characterized. Sequence analysis revealed that the DNA regions of MS14 shared the highest nucleotide identity (90.4%) to the genes in an uncharacterized region of strain AMMD. Interestingly, the product of the AMMD gene cluster has not been characterized. Taken together, the genomic locus identified in this study is a novel genetic element dedicated to biosynthesis of antifungal compound.

Conclusions

This study reveals that a genetic locus is responsible for the production of antifungal compound by strain MS14. Mutagenesis and bioassays demonstrate that the two NRPS genes (ORFs 5 and 6) contribute to the biosynthesis of the antifungal compound. The transcription of the two genes is regulated by *ambR2*, which encodes a putative LuxR type regulator. Further sequence analysis of the genes and preliminary chemical analysis indicate that the backbone of the antifungal compound is an oligopeptide that is synthesized via a NRPS mechanism. The study provides a firm base

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for future work aimed at characterizing the production and chemical composition of the antifungal compound. Currently, sequencing of the whole genomic region associated with the antifungal activity of MS14 is under way, as well as the elucidation of chemical structure of the compound. This work will provide important clues for development of pharmaceutical medicines or agricultural fungicides.

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Table 2.1 Primers used in this study

Primer	Sequence (5’-3’)
AmbR2F	5'-CCGCGGCGATCAGGTTGTA
AmbR2R	5'-CATGGAGTTCAGCAGATTGTTCGC
ORF2F	5'-GACTTTCATGCAGGCATAGGAGGG
ORF2R	5'-GATGATCGCCAGACGCTGTTGC
ORF3F	5'-CGGCGACGCGATTTCACTG
ORF3R	5'-CGGGGATTGCTTTCGATGCTG
ORF4F	5'-GAAGTGATACGCGCCCAGGAAC
ORF4R	5'-CATCTCGCCGCGGTGAAACA
ORF5F	5'-GTCGGTGACCAGCACGTTATTGT
ORF5R	5'-GTGCGTGCTGCTTCAGGTTGTT
ORF6F	5'-ACCCGTCGAGCAGGATGTGGT
ORF6R	5'-CGCGGCTTCGACTTTACCC
RecAF	5'-AAGATCGGCCAGGGCAAGGA
RecAR	5'-ACCATCGGGCATCGTGACCA
Bu16SF	5’-AGAGTTTGATCCTGGCTCAG
Bu16SR	5’-TACGGCTACCTTGTTACGACTT

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Table 2.2 The putative genes identified in the *ambR2* region

Gene or ORF	**Size (bp)**	**Homologue** [a]	**Identity (%)**	**Predicted Function** [b]
ambR2	891	bamb_6468	77	LuxR-type regulator
ORF2	1704	bamb_6469	90	Cyclic peptide transporter
ORF3	483	bamb_6470	82	Hypothetic protein
ORF4	657	bamb_6471	94	Glycosyl transferase
ORF5	9495	bamb_6472	88	Nonribosomal peptide synthetase
ORF6	9066	bamb_6473	89	Nonribosomal peptide synthetase

a: Homolog to the putative proteins of *Burkholderia ambifaria* AMMD (GenBank accession no. NC_008392); b: Predicted functions are based on annotation of strain AMMD.

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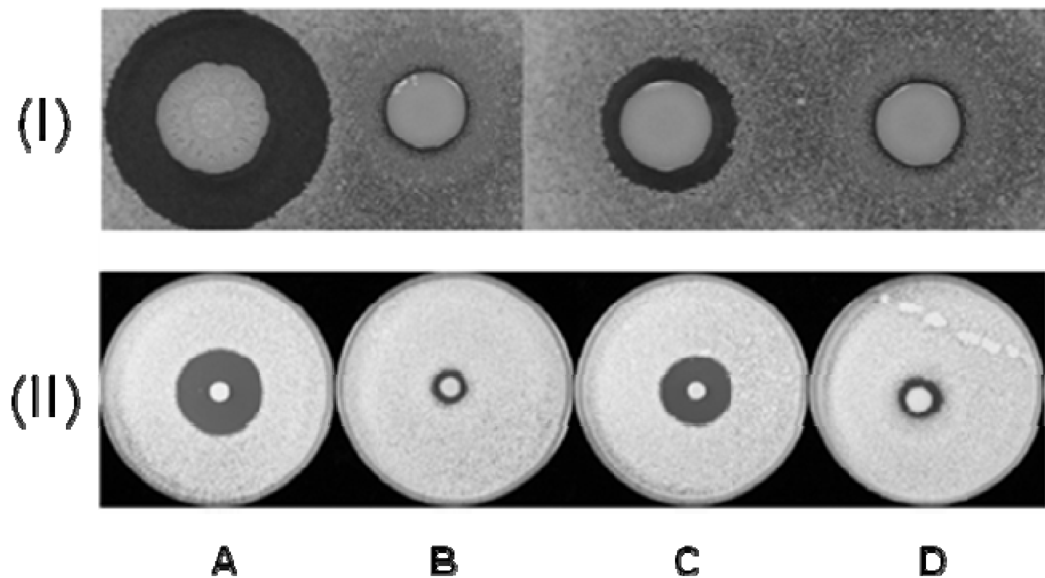

Figure 2.1 Plate bioassays of antifungal activities of Burkholderia contaminans MS14.

Plate bioassays of antifungal activities of Burkholderia contaminans MS14 with its mutants against the indicator fungus Geotrichum candidum. (I) Mutants of strain MS14. A: the wild type strain MS14; B: MS14MT13; C: MS14MT15; and D: MS14MT18. (II) Complementation of the mutant MS14MT15. A: the wild type strain MS14; B: MS14MT15; C: MS14MT15 (pGG2); and D: MS14MT15 (pMLS7).

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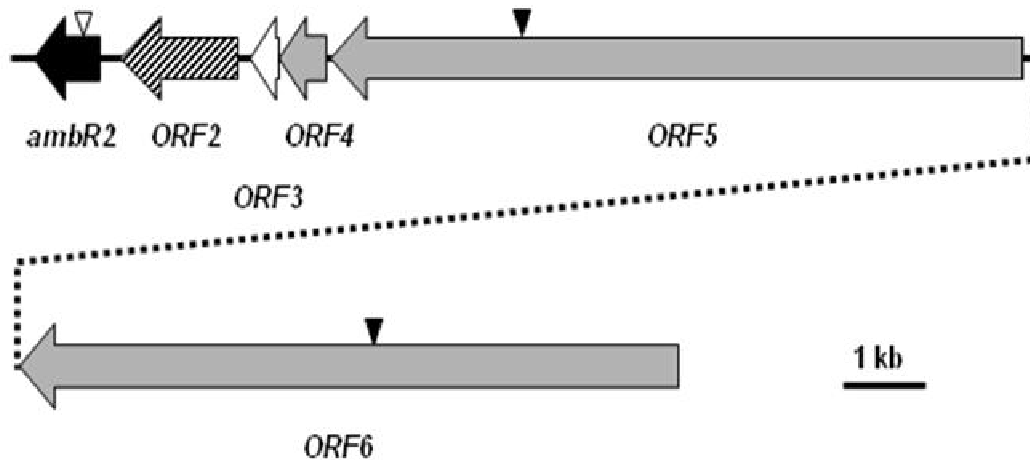

Figure 2.2 Gene organization of a 22.7-kb genomic region on the chromosome of *Burkholderia contaminans* MS14.

Vertical arrows indicate the insertion positions of the Tn*5* transposon. The open and solid vertical arrows represent reduction and elimination of antifungal activity, respectively.

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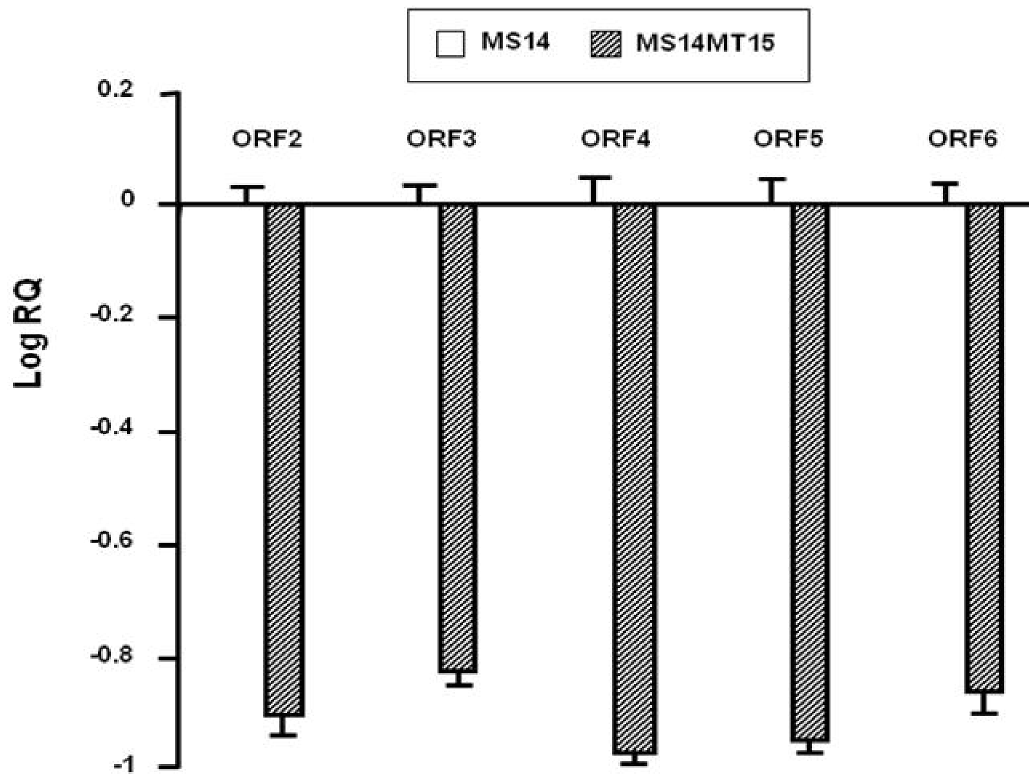

Figure 2.3 Expression of the ORFs identified in the genetic locus in the mutant MS14MT15.

Transcript levels of the tested ORFs are presented relative to the transcript levels in the wild type MS14. Mean values for three biological replicates are given, and error bars represent the standard errors of the means.

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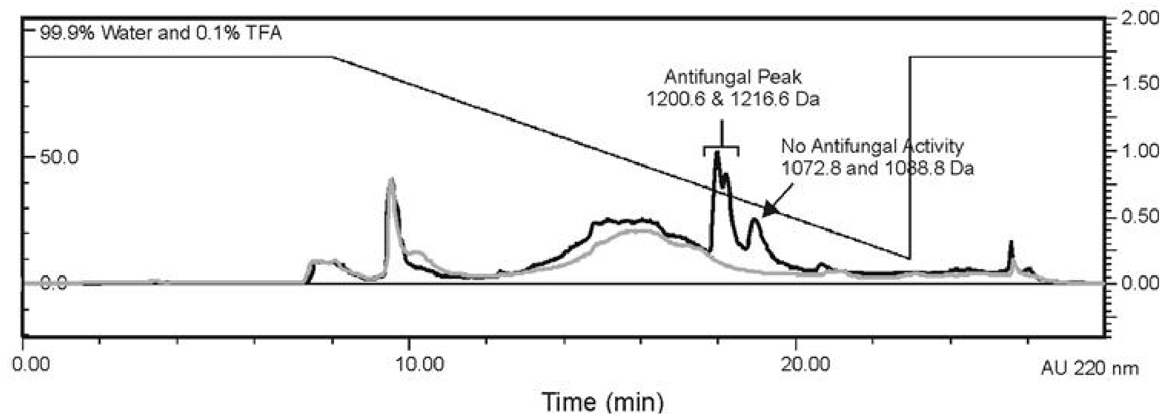

Figure 2.4 Overlaid RP-HPLC chromatogram for the extraction of antifungal compound producted by *Burkholderia contaminans* strain MS14.

Overlaid RP-HPLC chromatogram obtained from the extracts from both the wild type strain MS14 (black) and mutant strain MS14MT18 (gray). A doublet peak, which is unique to MS14, was determined to be the antifungal compound.

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CHAPTER III

AMBR1 IS A KEY TRANSCRIPTIONAL REGULATOR FOR PRODUCTION OF ANTIFUNGAL ACTIVITY OF *BURKHOLDERIA CONTAMINANS* STRAIN MS14

Introduction

Burkholderia bacteria are frequently isolated from the rhizosphere of crops, and are involved in growth promotion of plants and suppression of plant diseases (3,13). However, biological control strains of *Burkholderia* have not been clearly distinguished from human pathogenic strains, which has lead to the need for the reassessment of the risk of the registered *Burkholderia* strains for biological control (13). Analysis of the molecular mechanisms will provide useful clues to developing biofungicides instead of using living bacterial cells.

The LuxR-type regulatory genes are frequently identified in bacterial genomes (4,10). The LuxR/FixJ regulator family (PROSITE documentation: PDOC00542) consists of bacterial regulatory proteins that contain DNA-binding helix-turn-helix (HTH) domains at their C termini (8). Based on their functional mechanisms, these LuxR type proteins are grouped into two major subfamilies: response regulators and autoinducer-binding regulators (8). The response regulator subfamily includes the response regulators within two-component signal transduction systems, such as FixJ (2), and these proteins have a receiver domain, which is referred to as the acid pocket with highly conserved acidic residues in the N termini (14). The other subfamily is composed of autoinducer-binding regulators, such as LuxR, with seven highly invariant residues (5). These LuxR

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type regulators control a wide variety of biological functions (8), including production of secondary metabolites (10).

Burkholderia contaminans strain MS14 was isolated from a disease-suppressive soil and showed a broad range of antifungal activities to plant and human pathogens (12). A 22.7-kb genomic DNA fragment harboring six open-reading frames (ORFs) including a LuxR type regulatory gene *ambR2* is involved in biosynthesis of an antifungal oligopeptide in strain MS14 (7). In this study, further sequence analysis revealed the presence of another LuxR type regulatory gene, named *ambR1,* and three additional ORFs. The roles of *ambR1* in expression of the antifungal activity of MS14 were investigated.

Materials and methods

Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 3.1. *Burkholderia* strains were cultured at 28°C on nutrient broth–yeast (NBY) extract agar medium (22). Potato dextrose agar (PDA, Difco, Detroit, MI) was used for plate bioassays of *Burkholderia* strains to evaluate antifungal activities. Antibiotics (Sigma Chemical Co., St. Louis, MO) were added to media at the following concentrations: trimethoprim (50 μg mL^{-1}) and kanamycin (100 μg mL^{-1} for *E. coli* and 300 μg mL^{-1} for *Burkholderia* strains, as needed).

Genomic library screening and sequencing

To identify more genes associated with the production of antifungal compound of strain MS14, the fosmid 3C6 was identified from the MS14 genomic library using the 0.4-kb PCR product from pSL615 as described previously (7). DNA inserts of fosmids

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5F12 (7) and 3C6 were sequenced using a random shotgun approach (20). At least triple coverage of sequencing reactions was achieved, and the Lasergene software package (DNASTAR, Inc., Madison, WI) was used for generation of consensus sequences and multiple sequence analysis. InterProScan program was used for prediction of functional domains of proteins (16). NRPSpredictor was used for specificity prediction of adenylation domains in nonribosomal peptide synthetases (NRPSs) (17). Phylogenetic analyses were conducted in MEGA4 (21).

Bioassay for antifungal activities

B. contaminans strain MS14 and its mutants used in this study were evaluated for antifungal activities against *Geotrichum candidum* F-260 (6) using the PDA plate bioassays described previously (7). Three replicates for the plate bioassays were performed independently.

Random mutagenesis

To characterize additional genes dedicated to antifungal activity, the mutant MS14MT16 (Table 3.1) was generated and plasmid pSL616 was obtained using an EZ-Tn5 <R6Kγ*ori*/KAN-2>Tnp Transposome kit (Epicentre Biotechnologies, Madison, WI) as described previously (7).

Site-directed mutagenesis of the *ambR1* gene and plasmid construction for complementation assays

The wild-type *ambR1* gene was disrupted by the insertion of a kanamycin cassette into its ORF as described previously (10). A 4-kb *Pst*I fragment of fosmid 5F12 carrying *ambR1* was cloned into pBluescript II SK (Strategene, La Jolla, CA) resulting plasmid pGG4. Plasmid pGG4 was partially digested by *Bam*HI, blunted by T4 DNA

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polymerase, and religated to remove the *Bam*H1 restriction endonuclease site of pBluescript II SK, resulting plasmid pGG5. A 1.1-kb *Bam*H1 fragment of plasmid pBSL15 (1), which carries *nptII* without a transcriptional terminator, was inserted into the *Bam*H1 site of plasmid pGG5, which is located in the frame of *ambR1*, generating plasmid pGG6. A 5-kb *Pst*I fragment of plasmid pGG6 harboring the *ambR1* gene disrupted by insertion of *nptII* was cloned into pBR325 (15) at the *Pst*I site to generate pGG7.

Mutagenesis of the *ambR1* gene was conducted via a marker exchange procedure as described previously (10), resulting in the generation of the mutant MS14GG44 (Table 3.1). Southern blotting analysis and PCR were used to verify the double crossover mutants.

Plasmid pGG10 carrying the intact *ambR1* gene was constructed for a complementation assay of the mutant MS14GG44(*ambR1*::*nptII*). A 1.5-kb *Eco*R1 fragment carrying the intact *ambR1* gene of pGG4 was inserted into expression vector pMLS7 (9) to generate pGG10 for complementation assays. The empty vector pMLS7 was used as a control.

Constitutive expression of the *ambR2* gene in mutants MS14MT15 and MS14GG44

The intact ORF of *ambR2* was amplified using primers Eamb2F with *Bam*HI and Eamb2R with *Hin*dIII (Table 3.3) following a routine PCR method (18), and the product (1 kb) was ligated into pGEM T-Easy vector (Promega Corporation, Madison, WI) to generate pGG11. The 1-kb DNA fragment containing the intact *ambR2* gene in pGG11 was digested by *Bam*HI and *Hin*dIII, and cloned into expression vector pMLS7 to generate pGG14. Plasmid pGG14 was electroporated into cells of mutants MS14MT15

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and MS14GG44, respectively. Trimethoprim and kanamycin resistance colonies were confirmed to contain pGG14 by plasmid extraction and sequencing. Plate bioassays were used to evaluate the antifungal activity of the resulting cells.

Quantitative real-time PCR

To measure effects of mutations in *ambR1* and *ambR2* on the ORFs present in the genetic locus, transcription of the ORFs were analyzed using quantitative real-time PCR (Q-PCR) as described previously (4,7).

Results

Sequence analysis of the flanking regions of the 22.7-kb genomic DNA fragment

The flanking regions of the previously described 22.7-kb genomic fragment (7) were sequenced, revealing additional genes required for the production of antifungal compound. The assemblage of sequencing reads formed two contigs, which generated two consensus sequences flanking the 22.7-kb fragment: 3 kb downstream of the *ambR2* gene and 19.5 kb upstream of the ORF6. In combination of the 22.7 kb sequence, totally 45.2-kb genomic DNA fragment was sequenced and deposited into GenBank with the accession number: EU938698. The *ambR1* gene was identified in the 3-kb region, which is located downstream of *ambR2* and has an opposite transcriptional orientation to *ambR2* (Fig. 3.1). The *ambR1* gene was predicted to encode a LuxR type regulator (273 a.a.) and shared the highest similarity to bamb_6466 of *B. ambifaria* AMMD (GenBank accession number: NC_008392) (Table 3.2). Interestingly, no homolog to bamb_6467, a hypothetical protein located between bamb_6466 and bamb_6468 (the *ambR2* homolog) in the AMMD genome, was identified between *ambR1* and *ambR2*. Additionally, a

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partial ORF (ORF1) downstream of *ambR1* was predicted to encode the N terminus of a FAD linked oxidase domain-containing protein.

Three new ORFs (ORFs 7, 8, and 9) were identified upstream of ORF6 in the 19.5-kb flanking region (Table 3.2). Organization and orientations of the three ORFs are the same as those of the AMMD genome with an averaged amino acid identity of 91% (Fig. 3.1; Table 3.2). ORF7 was predicated to encode a NRPS (1306 a.a.) and to contain one module to catalyze the incorporation of an aromatic amino acid. The putative protein (538 a.a.) from ORF8 was predicted to contain a beta-lactamase domain. Interestingly, ORF9 was predicted to encode a multiple-domain protein (4469 a.a.). A beta-ketoacyl synthetase domain was found in the N terminus, and a NRPS module was identified in the C terminus, which was predicted to catalyze the incorporation of serine. Sequence analyses of pSL616 revealed the transposon in the mutant MS14MT16, which was defective in antifungal activity (Fig. 3.2), was located between nucleotides 9985-9986 of ORF9 relative to its translational start codon (Fig. 3.1). These data indicate that ORF9 might be involved in production of the oligopeptide in strain MS14.

The AmbR1 and AmbR2 proteins were predicted to be distinctive members of the LuxR family

A striking feature of this genetic region is the presence of two LuxR family regulatory genes, which are located adjacent to one another (Fig. 3.1). Blast search showed AmbR1 and AmbR2 proteins shared the highest amino acid similarities to the LuxR regulatory proteins, such as SyrF (42% and 33%, respectively) that is a transcriptional regulator crucial for syringomycin production in *Pseudomonas syringae* pv. *syringae* (10). The deduced AmbR1 protein exhibited 36% identity to AmbR2. The HTH DNA binding domains were identified at the C termini of AmbR1 and AmbR2.

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Neither autoinducer-binding domain nor response regulatory domain was found in the two protein sequences using the InterProScan program (16), which indicates that AmbR1 and AmR2, like SyrF (10), may have different regulatory mechanisms from current members of the LuxR/FixJ protein family.

Effect of mutation in *ambR1* on antifungal activity

A nonpolar mutation was constructed by insertion of an *nptII* cassette into *Bam*HI of *ambR1*, and the mutant MS14GG44 was generated by marker exchange mutagenesis. The mutant exhibited almost negligible antifungal activity towards *G. candidum* (inhibitory zone radius ± SEM: 0.33 ± 0.33 mm) (Fig. 3.2), which is similar to the mutants MS14MT13, MS14MT18 (7), and MS14MT16 (Fig. 3.1). As expected, the wild-type strain MS14 showed a strong inhibition to the fungus (13.00 ± 0.58 mm). The wild-type level of antifungal activities against *G. candidum* was observed for MS14GG44 complemented *in trans* with plasmid pGG10 (12.67 ± 0.33 mm), while the introduction of the empty vector pMLS7 did not affect the phenotype of MS14GG44 (Fig. 3.2). These results reveal the essential role of *ambR1* in the expression of antifungal activity of strain MS14.

Functional *ambR1* and *ambR2* were required for transcription of all the ORFs except ORF1

To test whether the mutation in *ambR1* affected transcription of the ORFs, Q-PCR analyses were performed with RNAs isolated from strain MS14 and the mutant MS14GG44. As expected, significant differences ($P < 0.05$) of expression levels of ORFs 2-9 in the mutant MS14GG44 were observed as compared with those in the wild-type strain MS14. Transcript levels of the ORFs in mutants MS14GG44 were reduced

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13.2 fold on average (Fig. 3.3A). A previous study demonstrated that *ambR2* positively regulated transcription of ORFs 2-6 (7). As expected, expression levels of ORFs 7, 8 and 9 in the mutant MS14MT15 were significantly reduced 27.2 fold on average as compared to strain MS14 (Fig. 3.3B). In contrast, transcription of ORF1 was not significantly affected by mutations in either *ambR1* or *ambR2*, indicating that the ORF is not regulated by either of them. These data reveal that *ambR1* and *ambR2* gene products act to promote, either directly or indirectly, transcription of ORFs 2-9.

Regulatory relationship of AmbR1 and AmbR2

To test the relationship between the two regulators, the transcriptional levels of the *ambR1* and *ambR2* genes in strain MS14 and the mutants MS14GG44 and MS14MT15 were measured by Q-PCR (Fig. 3.3C). The RNA level of *ambR2* in mutant MS14GG44 was significantly reduced 25 fold as compared with MS14. The full expression of *ambR2*, which was equivalent to levels in MS14, was restored when plasmid pGG10 containing the intact *ambR1* gene was introduced into cells of mutant MS14GG44. In contrast, no significant difference ($P < 0.05$) of transcription of *ambR1* was observed among strains MS14, MS14MT15, and MS14MT15[pGG2]. These data demonstrate that a functional *ambR1* gene is essential for transcription of the regulator gene *ambR2*, while *ambR2* has no effect on the transcription of *ambR1*.

The intact *ambR1* gene regulated by the S7 ribosomal protein promoter (9) in plasmid pGG14 was transformed into the mutants MS14MT15 and MS14GG44, and expressed constitutively. As expected, the strain MS14MT15[pGG14] resulted in recovery of the antifungal activity against *G. candidum* to the wild-type level (Fig. 3.2). However, the strain MS14GG44[pGG14] did not restore its antifungal activity. These

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results suggest that constitutive expression of *ambR2* could not restore the function of *ambR1* in the production of the antifungal activity and both *ambR1* and *ambR2* have direct regulatory roles for the activity.

Discussion

The crucial function of the *ambR1* regulatory gene in production of antifungal activity of strain MS14 is attributed to the following factors. First, the antifungal activity of the *ambR1* mutant MS14GG44 was eliminated while the *ambR2* mutant MS14MT15 was reduced approximately 80% (7), indicating that *ambR1* plays a larger role than *ambR2* in production of the antifungal activity. Second, like *ambR2*, *ambR1* positively regulates transcription of ORFs 5, 6, 7 and 9, which were predicted to encode putative NRPSs that are required for antifungal activity of strain MS14. Both functional *ambR1* and *ambR2* genes are required for a full expression of ORF4, which codes for a putative cyclic peptide transporter and is believed to be responsible for the secretion of the oligopeptide. Third, the *ambR1* gene controls transcription of *ambR2* and constitutive expression of *ambR2* cannot restore the *ambR1* phenotype. This suggests that *ambR1* is not only located at a higher position in a regulatory cascade, but also plays a direct role in regulation of the production of the antifungal activity. Such a regulatory relationship of *ambR1* and *ambR2* is the same as that of *salA* and *syrF* (10). It is predicted that the *ambR1* gene, like the *salA* regulatory gene in syringomycin and syringopeptin production, encodes a major transcriptional regulator governing the whole gene cluster for the production of the antifungal compound in MS14. In fact, in addition to the *syr-syp* genes, *salA* also regulates the production of another secondary metabolite, syringolin,

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whose biosynthesis genes are located outside the *syr-syp* gene clusters (11). It remains to be explored if *ambR1* is required for the production of other secondary metabolites.

The genes involved in production of NRPS oligopeptides are frequently clustered in bacterial genomes and co-regulated by transcriptional factors (10,19). The conserved ORF1 (partial) encodes the N terminus of a FAD linked oxidase domain-containing protein and its transcription is not regulated by either *ambR1* or *ambR2*, which implies the gene may not be part of the gene cluster required for antifungal activity, and *ambR1* is the last gene at the left border of the gene cluster. Previous studies revealed that two NRPS genes, which were predicted to catalyze four amino acids, were required for antifungal activity of strain MS14 (7). In this study, two additional NRPS genes, which were predicted to carry two catalytic modules, were characterized. The antifungal oligopeptide is predicted by 2D NMR to contain eight amino acids (Smith, L. Lu, S.-E and Gu, G., unpublished). Therefore, more genes involved in production of the antifungal oligopeptide are expected to exist upstream of ORF9. Characterization of these genes will be part of future efforts to further our understanding of this novel gene cluster.

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Table 3.1 Bacterial strains and plasmids

Strains or plasmids	Relevant characteristics	Source
Escherichia coli		
JM109	*recA1*, *endA1*, *gyr*A96, *thi*, hsdR17, *sup*E44, *rel*A1, Δ(lac-proAB)/F' [traD36, proAB+, *lacIq*, *lacZ*ΔM15]	Promega
Burkholderia contaminans		
MS14	Wild type strain	(7)
MS14MT15	*ambR2*::Tn5 derivative of MS14; Km^r	(7)
MS14MT16	*ORF9*::Tn5 derivative of MS14; Km^r	This study
MS14GG44	*ambR1*::*nptII* derivative of MS14; Km^r	This study
Plasmid /fosmid		
pSL615	EZ-Tn5 carrying the ~0.5 kb genomic DNA of MS14MT15; Km^r	(7)
pSL616	EZ-Tn5 carrying the ~8 kb genomic DNA of MS14MT13; Km^r	This study
pBluescript II SK	Cloning vector; Ap^r	Strategene
pBR325	Cloning vector; Cm^r, Tc^r, Ap^r	(15)
pMLS7	Expression vector of *Burkholderia*; Tp^r	(9)
pGEM-T Easy	Cloning vector; Ap^r	Promega
pBSL15	Kanamycin resistance gene cassette; Km^r	(1)
pGG2	pMLS7 carrying 2-kb *Pst*I fragment harboring the intact *ambR2* gene; Tp^r	(7)
pGG4	pBluescript II SK carrying the 4 kb *Pst*1 fragment of 5F12 containing *ambR1*; Ap^r	This study
pGG5	pGG4 lacking the *Bam*HI site of the pBluescript II SK ploylinker; Ap^r	This study

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Table 3.1 (continued)

pGG6	pGG5 with the *nptII* insertion in *ambR1*; Km^r, Ap^r	This study
pGG7	pBR325 carrying the 5-kb *Pst*1 fragment containing *ambR1* disrupted by *nptII*; Cm^r, Tc^r, Km^r	This study
pGG10	pMLS7 carrying 1.5-kb *Eco*RI fragment harboring the intact *ambR1* gene; Tp^r	This study
pGG11	pGEM-T Easy carrying 1-kb PCR product containing the intact *ambR2* gene; Ap^r	This study
pGG14	pMLS7 carrying 1-kb *Bam*HI and *Hin*dIII fragment containing the intact *ambR2* gene; Tp^r	This study
5F12	pCC1FOS carrying 40-kb genomic DNA of MS14; Cm^r	(7)
3C6	pCC1FOS carrying 40-kb genomic DNA of MS14; Cm^r	This study

* Km^r: Kanamycin resistance; Ap^r: Ampicillin resistance; Tp^r: trimethoprim resistance; Cm^r: Chloramphenicol resistance

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Table 3.2 The putative genes identified from the flanking regions of the 22.7-kb genomic DNA fragment

Gene or ORF	**Size (bp)**	**Homolog** [a]	**Identity (%)**	**Predicted Function** [b]
ORF1(partial)	1175	bamb_6465	93	FAD linked oxidase domain protein
ambR1	822	bamb_6466	89	LuxR-type regulator
ORF7	3921	bamb_6474	90	Nonribosomal peptide synthetase
ORF8	1617	bamb_6475	93	Beta-lactamase domain protein
ORF9	13410	bamb_6476	90	Beta-ketoacyl synthetase and Nonribosomal peptide synthetase

a: Homolog to the putative proteins of *Burkholderia ambifaria* AMMD^T (GenBank: NC_008392); b: Predicted functions are based on the annotation of strain AMMD.

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Table 3.3 Primers used in this study

Primer	Sequence	Source
ORF1F	5'-TCACGATCCGCTTGACGAGAT	This study
ORF1R	5'-CAGCAAGCCGTTCCTGTCG	This study
AmbR1F	5'-GGGCGGCGGATTTCAGTG	This study
AmbR1R	5'-GAGCGGCAGCAGCGTATC	This study
AmbR2F	5'-CCGCGGCGATCAGGTTGTA	(7)
AmbR2R	5'-CATGGAGTTCAGCAGATTGTTCGC	(7)
ORF2F	5'-GACTTTCATGCAGGCATAGGAGGG	(7)
ORF2R	5'-GATGATCGCCAGACGCTGTTGC	(7)
ORF3F	5'-CGGCGACGCGATTTCACTG	(7)
ORF3R	5'-CGGGGATTGCTTTCGATGCTG	(7)
ORF4F	5'-GAAGTGATACGCGCCCAGGAAC	(7)
ORF4R	5'-CATCTCGCCGCGGTGAAACA	(7)
ORF5F	5'-GTCGGTGACCAGCACGTTATTGT	(7)
ORF5R	5'-GTGCGTGCTGCTTCAGGTTGTT	(7)
ORF6F	5'-ACCCGTCGAGCAGGATGTGGT	(7)
ORF6R	5'-CGCGGCTTCGACTTTACCC	(7)
ORF7F	5'-GTCGGTGACCAGCACGTTATTGT	This study
ORF7R	5'-GTGCGTGCTGCTTCAGGTTGTT	This study
ORF8F	5'-ACCCGTCGAGCAGGATGTGGT	This study
ORF8R	5'-CGCGGCTTCGACTTTACCC	This study
ORF9F	5'-TCGCGGTTGTAGTGCCAGGTC	This study
ORF9R	5'-CCGGTGCGCTTCAACTATCTCG	This study
RecAF	5'-AAGATCGGCCAGGGCAAGGA	(7)
RecAR	5'-ACCATCGGGCATCGTGACCA	(7)

* Underlined letters represent restriction endonuclease sites.

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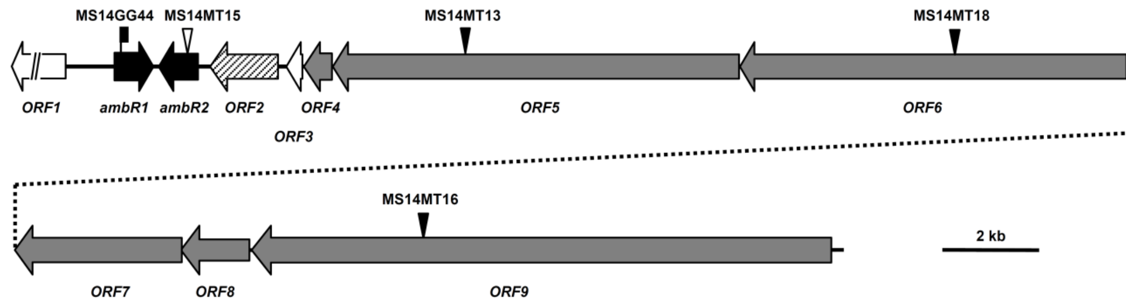

Figure 3.1 Map of a 45.2-kb genomic region on the chromosome of *Burkholderia contaminans* MS14.

The positions and orientations of the known genes and potential ORFs are shown as horizontal arrows. The discontinuous arrow represents a partial ORF. Vertical arrows and the flag indicate the insertion positions of the Tn*5* transposon and the *nptII* cassette, respectively. The open and solid vertical arrows/flag represent reduction and elimination of antifungal activity, respectively.

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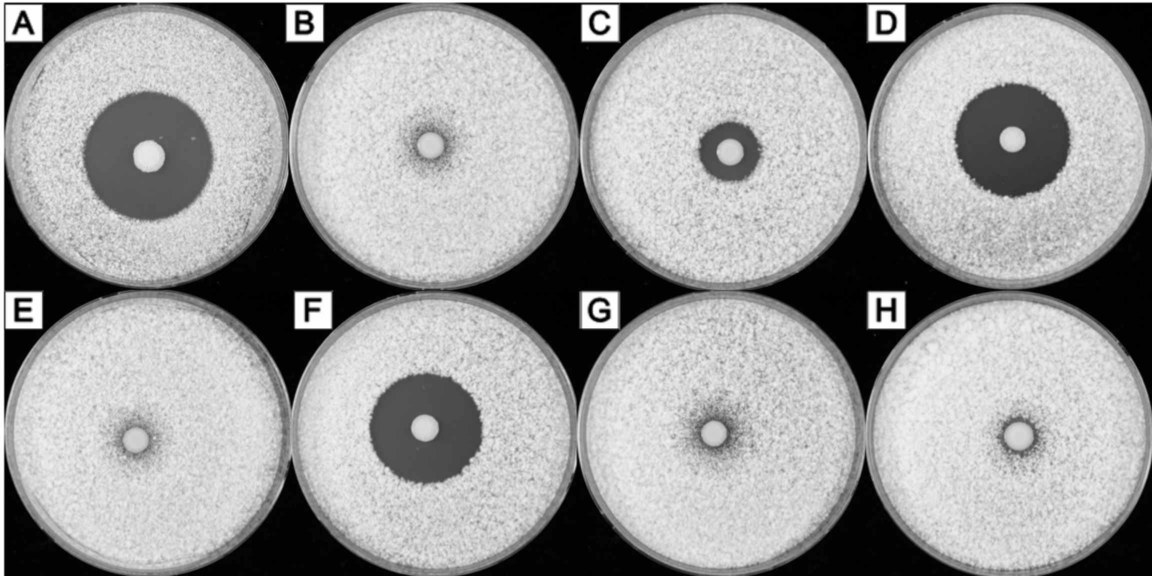

Figure 3.2 Plate bioassays for antifungal activities of *Burkholderia contaminans* MS14 and its mutants.

Potato dextrose agar plates were inoculated with each of the strains and incubated for 3 days at 28°C. The plates were oversprayed with the indicator fungus *Geotrichum candidum* and further incubated overnight. A: The wild-type strain MS14; B: MS14MT16(*ORF9*::Tn*5*); C: MS14MT15(*ambR2*::Tn*5*); D: MS14MT15 [pGG14]; E: MS14GG44(*ambR1*::*nptII*); F: MS14GG44 [pGG10]; G: MS14GG44 [pGG14]; and H: MS14GG44 [pMLS7].

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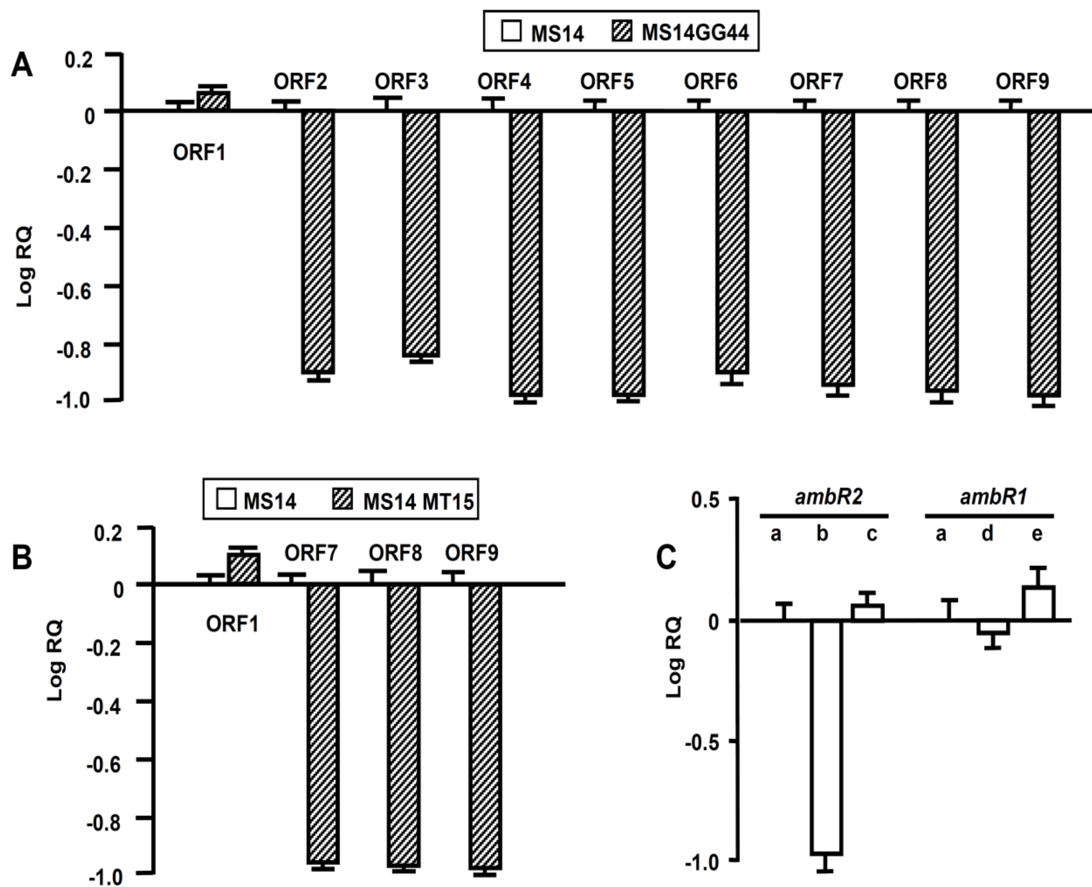

Figure 3.3 Relative transcription of the ORFs identified in the 45.2-kb genetic DNA fragment.

Transcript levels of the tested ORFs are presented relative to those in the wild-type strain MS14. The C_T values of tested *ambR1* and *ambR2* genes and the other ORFs were corrected by the housekeeping gene *recA* as follows: $\Delta C_T = C_{T(\text{tested})} - C_{T(recA)}$. The relative quantification (RQ) values were calculated by the formula RQ = $2^{[\Delta C_{T(\text{mutant})} - \Delta C_{T(\text{wild type})}]}$. Three replicates of Q-PCR were conducted independently. Vertical bars indicate standard error of the means. A: Effect of the *ambR1* mutation on transcription of the ORFs identified. B: Effect of the *ambR2* mutation on transcription of the newly identified ORFs from the flanking regions of the 22.7 kb fragment. C: Effect of mutations in *ambR1* and *ambR2* on transcription of the two genes: a, MS14; b, MS14GG44(*ambR1*::*nptII*); c, MS14GG44[pGG10]; d, MS14MT15(*ambR2*::Tn*5*); e, MS14MT15[pGG2].

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CHAPTER IV

CHARACTERIZATION OF THE RIGHT BORDER OF THE *OCC* GENE CLUSTER ASSOCIATED WITH THE ANTIFUNGAL ACTIVITY OF *BURKHOLDERIA CONTAMINANS* STRAIN MS14

Introduction

Burkholderia bacteria, which distribute widely in nature, are reported to be important for the plant growth promotion (4). However, taxonomic distinctions have not enabled biological control strains to be clearly distinguished from human pathogenic strains, which has lead to the reassessment of the risk of the registered *Burkholderia* strains as biological control agents (24). For example, some strains of *B. contaminans* were isolated from sputum and blood samples of debilitated patients such as cystic fibrosis patients (34), and some of them showed significant antifungal activity (11). Analysis of the genomic structures and molecular mechanisms will benefit the development of safer biologically based management and eliminate the potential harmfulness the living bacteria may bring.

Till now, the whole genomic sequences of 22 strains of *Burkholderia* are available in NCBI (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). According to the annotation of the ExPASy Proteomics Server, eight of them are human pathogens including three strains of *B. cenocepacia*, three strains of *B. pseudomallei* and two strains of *B. mallei*. *B. cenocepacia* is the opportunistic pathogen of human cystic fibrosis or chronic granulomatous diseases (22). *B. pseudomallei* and *B. mallei* are the causal agents

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of melioidosis and glanders diseases, respectively (3,15). The human pathogenesis of two strains, *B. ambifaria* AMMD and *B. multivorans* strain ATCC17616, is still unknown. The other 12 strains were considered to be not relative to the human pathogenesis, such as *B. lata* strain 383 (http://www.expasy.ch/sprot/hamap/BURM1.html).

Nonribosomal peptide synthetase (NRPS) and polyketide synthetase (PKS) are large multimodular enzymes, which are involved in natural product synthesis in many microorganisms (5). NRPS, involved in the biosynthesis of oligopeptide, is grouped by active sites termed modules, in which each module is required for catalyzing one single cycle of product length elongation. The order and number of the modules of an NRPS protein are mainly followed by the “collinearity rule” (10). There are three main domains in each module: the adenylation (A) domain, responsible for amino acid recognition and adenylation at the expense of ATP; the thiolation (T) domain, carrier of thioesterified amino acid intermediates; and the condensation (C) domain, catalyzing peptide bond formation between two consecutive amino acids (5). The epimerization (E) domain as one of the modification domains catalyzes the conversion of L-amino acids to their D-isomers (23). Cyclization and release of the peptide product are catalyzed by the C-terminal thioesterase (Te) domain (29). Polyketide synthetases are a family of enzymes or enzyme complexes that produce polyketides, a large class of secondary metabolites, in bacteria, fungi, plants, and a few animals lineages (16). The biosyntheses of polyketides share striking architectural and organizational similarities with nonribosomal peptide biosynthesis, and their modules can be integrated to produce the hybrid NRPS-PKS products. Some pharmaceutical antimicrobial agents, such as the precursor of penicillin and erythromycin, are synthesized through these mechanisms (6,33).

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ATP-binding cassette (ABC) transporters are a large superfamily of integral membrane proteins that carry various substrates across cellular membranes, which are involved in the export or import of a wide variety of substrates ranging from ions to large polypeptides (14). Extensive studies showed ABC transporters are one of major groups of secretion machinery associated with transportation of bacterial secondary metabolites, such as drugs and antibiotics (7).

B. contaminans strain MS14 showing a broad range of antifungal activities to plant and animal pathogens was isolated from a disease-suppressive soil (21). Production of an octapeptide, named as occidiofungin, is responsible for the antifungal activities of strain MS14 (19). Occidiofungins, composed of eight amino acids, are two structure-related antifungal compounds, with the masses of 1119.5 and 1215.5 Da, respectively (Fig. 4.2a). A 45.2-kb genomic DNA fragment harboring 11 open-reading frames (ORFs) including the bisoythetase genes *occA*, *occB*, *occC*, and *occD*, two LuxR regulatory genes *ambR1* and *ambR2*, and the ABC transporter gene *occT* (11,12) was identified. Analysis of transcription demonstrated that both *ambR1* and *ambR2* are essential for the expression of all the ORFs except for ORF1 and the production of the antifungal activity. In this study, the right border of the *occ* gene cluster was sequenced, and further sequence analysis revealed the presence of another seven additional ORFs, which included *ORF11*(*occE*), another NRPS gene, and *ORF15*, a putative thioesterase.

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Materials and Methods

Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are described in Table 4.1. *Escherichia coli* strain JM109 was grown in Luria-Bertani medium at 37°C (28). *Burkholderia* strains were cultured at 28°C on nutrient broth–yeast (NBY) extract agar medium (35). When required, antibiotics were added at the following concentrations: ampicillin (100 µg ml^{-1}), trimethoprim (50 µg ml^{-1}), chloramphenicol (12.5 µg ml^{-1}), and kanamycin (100 µg ml^{-1} for *E. coli* and 300 µg ml^{-1} for the MS14 mutants).

Bioassay for antifungal activities

B. contaminans strain MS14 and its mutants used in this study were evaluated for antifungal activities against *Geotrichum candidum* F-260 using potato dextrose agar (PDA, Difco, Detroit, MI) plate bioassays described previously (11). Three replicates for the plate bioassays were performed independently.

Random mutagenesis, genomic library screening and sequencing

To identify more genes associated with production of antifungal compound of strain MS14, the mutant MS14MT24 (Table 4.1) was generated and plasmid pSL604 was obtained using an EZ-Tn5™ <R6Kγori/KAN-2>Tnp Transposome™ Kit (Epicentre Biotechnologies, Madison, WI) as described previously (11). Fosmid 4G5 was identified from the MS14 genomic library using the 1-kb PCR product from pSL624 as described previously (11). DNA inserts of fosmid 4G5 was sequenced using a random shotgun approach (31). At least triple coverage of sequencing reactions was achieved, and the Lasergene software package (DNASTAR, Inc., Madison, WI) was used for generation of consensus sequence. Bacterial ORFs were subsequently predicted by the Softberry

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FGENESB program (Softberry, Inc., Mount Kisco, NY), and the identified ORFs were analyzed using Blastx in the NCBI database. Putative promoter sequences were identified by the Softberry BPROM program. The InterProScan program was used for prediction of functional domains of proteins (26). NRPSpredictor (27) and the NPRS-PKS web-based software (2) were used for specificity prediction of adenylation domains in nonribosomal peptide synthetases (NRPSs). The program "IslandPick" was used to analyze the sequence characteristics as genomic islands (17).

RNA extraction and quantitative real-time PCR

Total RNA of strain MS14 and its mutants were extracted using an RNeasy Protect Bacteria kit (Qiagen, Valencia, CA) as recommended by the manufacturer. Transcription of the ORFs was analyzed using quantitative real-time PCR (Q-PCR) as described previously (10,11). Three replicates of Q-PCR were conducted independently, and statistical significant differences were determined for the log values of relative quantification by analysis of variance ($P < 0.05$) followed by the Bonferroni and Dunnet post hoc multiple comparisons (ANOVA, SAS Institute, Inc., NC, USA).

Site-directed mutagenesis of the genes *occT, occE* and *ORF15*

The wild-type *occT, occE* and *ORF15* genes were disrupted by the insertion of a kanamycin cassette into their open reading frames, respectively, as described previously (20). Plasmids pBR325 and pBSL15 were digested by *Eco*RI, blunted by T4 DNA polyermerase, and self ligated to generate plasmids pBR325[R1] and pBSL15[R1] without *Eco*RI (Table 4.1) (1). To mutate *occT*, the 2.1-kb fragment of the *occT* gene of strain MS14, obtained by PCR using the primers MoccTF containing the *Hin*dIII site and MoccTRA with the *Eco*RV site (Table 4.3), was digested with *Hin*dIII and *Eco*RV and

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inserted into pBR325[R1], resulting in plasmid pGG25. A 1.1-kb *Eco*RI fragment containing the *nptII* gene without a transcriptional terminator from pBSL15 was inserted into pGG25 at the *Eco*RI site, which is located in the frame of *occT*, to disrupt transcription of *occT*, resulting in pGG26. Mutagenesis of the *occT* gene was conducted via a marker exchange procedure as described previously (20), to generate the mutant MS14SL16 (Table 4.1). PCR analysis and sequencing were used to verify the double crossover mutants. Mutations of *occE* and *ORF15* were generated using a similar mutagenesis strategy. The 4-kb and 1-kb fragments of the *occE* and *ORF15* genes, obtained by PCR using two pairs of primers MoccEF and MoccER (for *occE*), MORF15F and MORF15R (for *ORF15*, Table 4.3), were cloned into the pGEM-T Easy Vector System I (Promega Corporation, Madison, WI) resulting in plasmids pGG20 and pGG15. The *nptII* gene was inserted into pGG20 at *Bam*HI and pGG15 at *Sma*I, generating plasmids pGG21 and pGG16, respectively. The 5-kb *Eco*RI fragment of pGG21 harboring the *occE* gene disrupted by insertion of *nptII* was cloned into pBR325 (25) at the *Eco*RI site to generate pGG22. The 2-kb *Eco*RI fragment of pGG16 harboring the *ORF15* gene disrupted by insertion of *nptII* was cloned into pBR325 (25) to generate pGG17. Mutagenesis of the *occE* and *ORF15* genes were conducted to generate the mutants MS14GG78 and MS14GG82, respectively, with the marker exchange as described above.

Complementation of *occE* mutant MS14GG78

To obtain the intact wild type gene *occE*, which was disrupted in the mutant MS14GG78, a 4.5-kb fragment was amplified by PCR using primers EoccEF and EoccER, both of which contain the *Hin*dIII site (Table 4.3), and cloned into the pGEM-T

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Easy Vector, resulting in the plasmid pGG23. The presence of the intact *occE* gene in the 4.5-kb DNA fragment was verified by sequencing. The 4.5-kb *Hin*dIII fragment harboring the intact *occE* gene was inserted into the *Burkholderia* gene expression vector pMLS7 (18), to generate the plasmid pGG24. The plasmid pGG24 were electroporated into cells of the mutant MS14GG78, an *occE* mutant (Table 4.1). Colonies acquiring the trimethoprim resistance were confirmed to contain the plasmid construct pGG24 by plasmid extraction and restriction enzyme digestion. Complementation experiments were conducted using the plate assays to evaluate antifungal activity against *G. candidum* as described previously (11).

Results and discussion

Sequence analysis of the right border of the *occ* gene cluster

The upstream region of *ORF9* of the previously described 45.2-kb genomic fragment (12) was sequenced through the sequencing of the fosmid 4G5, and seven new ORFs (*ORFs 10-16*) were identified in this region (Fig. 4.1; Table 4.2). In combination of the 45.2-kb sequence, a totally 58.2-kb genomic DNA fragment, named as the *occ* gene cluster, was obtained and deposited into GenBank with the accession number: EU938698. Organization and orientations of the *ORFs 10-15* are the same as those of the *B. ambifaria* strain AMMD genome with an averaged amino acid identity of 91%, and *ORF16* has the highest similarity (93%) to its homolog C7511 of *B. lata* strain 383 (Fig. 4.1; Table 4.2), while its homolog was not found in the genome of strain AMMD with BLAST. The putative protein (1107 a.a.) from *ORF10* was predicted to encode a short-chain dehydrogenase. *ORF11* (*occE*) was predicated to encode a NRPS (1475 a.a.) and to contain the first module to catalyze Asn (BHN1) amino acid of occidiofungin (Fig.

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4.2). *ORFs 12, 13,* and *14* were predicted to encode oxygenase, transaminase and epimerase respectively. Putative protein sequence of *ORF15* is 219 amino acids in length and carries a Te domain. Sequence analysis of pSL624 revealed the transposon in the mutant MS14MT24 defective in antifungal activity (Fig. 4.4) was located between nucleotides 12982-12983 of *ORF9* relative to its translational start codon (Fig. 4.1). Putative promoters were identified at the upstream of *ORF1*, *ambR1*, *ORF13* and *ORF16* and their locations are shown in Fig. 4.1.

Sequence analysis revealed that the *occ* gene cluster, excluding *ORF1* and *ORF16*, only shared high similarity (99% nucleotide coverage and 91% identity) to an uncharacterized DNA region of *B. ambifaria* strain AMMD. The gene cluster was not found in other *Burkholderia* strains available in GenBank (nucleotide coverage < 24%), including some identified opportunistic human pathogens *B. cenocepacia* strain J2315, AU1054, HI2424, *B. pseudomallei* strain 668, 1710b, 1106a, and *B. mallei* strain NCTC 10247, NCTC 10229. The sequences of the flanking regions of the *occ* gene cluster in strain MS14 share highest identities (89% nucleotide identity) with the homologs C7522 (left) and C7511 (right) in chromosome 3 of *B. lata* 383. The average G+C content of the *occ* gene cluster is 67.74%, which is closer to those of flanking region sequences in strain AMMD (left: bam_6475, 68.5% and right: bam_6483, 65.89%) but different from those of strain MS14 (55.16% left and 61.81% right). Besides, sequence analysis of the program "IslandPick" indicated that the uncharacterized exclusive homolog of the *occ* gene cluster in strain AMMD possessed the characteristics as a genomic island. These data suggest that the *occ* gene cluster, as a potential genomic island, may be horizontally transferred from a strain similar to *B. ambifaria* AMMD and integrated into a strain similar to *B. lata* 383. More importantly, the absence of the gene cluster in clinical

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strains of *Burkholderia* indicated that occidiofungin, the antifungal compound produced by the *occ* gene cluster of strain MS14, is not involved in the potential human pathogenesis. This finding has provided insights for the usage of occidiofungin, and clues for development of pharmaceutical medicines and agricultural fungicides.

Functional analysis of the occidiofungin synthetases

Based on the analogy to the known NRPSs, OccA, OccB, OccC, OccD, and OccE were predicted to be composed of two (8 and 7), two (6 and 5), one (4), two (3and 2), and one (1) modules, respectively (Fig. 4.2b). A detailed analysis of OccA - OccE revealed that the six modules encoded by OccA, OccB, OccC, OccD and the first module of OccE have in common the three major domains known as the condensation, adenylation, and thiolation domains. Among the eight amino acids of occidiofungain, Asn7 and Ser8 predicted to be catalyzed by OccA matched the prediction (Fig. 4.2). There were no confirmed modular prediction for OccB and OccE, which were predicted to catalyze Lys5, Gly6 and Ans1 (BHN1), all of them could find their corresponding homologs through the NPRS-PKS web-based software. OccC is predicted to catalyze the addition of Tyr-D with the 38% identity and 55% similarity. The C-terminal part of OccD is predicted to catalyze the production of Ser with the highest identity of 54% and similarity of 67%, or Trp with 40% identity and 55% similarity.

No match of amino acid substrate was found for the first half of the synthetase OccD from the current NRPS database. According to the position of the novel amino acid module 2 (NAA2) in the occidiofungin and the sequence characteristics of the *occD* gene, the fragment encoding the modules for the production of NAA2 was located at the N-terminal part of *occD*. The *occD* gene, which is essential for occidiofungin

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biosynthesis (see below), encodes a hybrid NRPS-PKS protein containing domains characteristic of both NRPSs and PKSs. Residing at the N terminus of *occD* are two PKS modules, which were predicted to synthesize two keto acids (malonate). To produce NAA2, it is hypothesized that, the putative transaminase encoded by *ORF13* transfers an amino group to one of the keto acids for the formation of the novel amino acid. All of these results indicated that occidiofungin might be produced through a novel hybrid NRPS-PKS mechanism. Collectively, these genetic analyses basically match the backbone structure of occidiofungin except for the No. 5 position of the oligopeptide.

Modifying genes for the production of occidiofungin

ORFs 12-15 in the left border of the *occ* gene cluster, were predicted to encode four putative modification enzymes involving in production of occidiofungin. The putative protein encoded by *ORF12* as an oxygenase, was predicted to introduce one atom of oxygen to Asn1 to form BHN1 or work for the formation of BHT4. ORF13, as a transaminase, was predicted to transfers an amino group for the formation of NAA2. Based on the analyses of NPRS-PKS web-based software, Tyr4 and Ser8 were predicted to be D formation. None of the eight modules, however, in the *occ* gene cluster harbors an internal E-domain for the conversion of the L to the D form, and the NAD-dependent epimerase domain was identified at *ORF14*, which suggest that this external epimerase is responsible for the conversion of the amino acid conformation L and D. Sequence analysis revealed that two putative TE domains were located at *ORF15* and the C-terminal region of *occA*, respectively, which were predicted to be important for the termination and cyclization of the production of occidiofungin. The putative glycosyl

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transferase encoded by *ORF4*, was predicted to catalyze the transfer of xylose for the formation of NAA2 (11).

Disruption of the NRPS gene *occE* eliminated the antifungal activity of strain MS14

A nonpolar mutation was constructed by insertion of an *nptII* cassette into *Bam*HI of *occE*, and the mutant MS14GG78 was generated by marker exchange mutagenesis. PCR analysis demonstrated that *occE* was disrupted by insertion of *nptII* in the genome of the mutant. The mutant exhibited negligible antifungal activity towards *G. candidum* (inhibitory zone radius ± SEM: 0.33 ± 0.33 mm), which is similar to the mutants MS14MT13, MS14MT18 (11), MS14MT16 (12), and MS14MT24 (Fig. 4.4). As expected, the wild-type strain MS14 showed a strong inhibition to the fungus (13.00 ± 0.58 mm). The wild-type level of antifungal activities against *G. candidum* was observed for MS14GG78 complemented *in trans* with plasmid pGG24 (12.67 ± 0.33 mm). As expected, the presence of the empty vector pMLS7 had no effect on antifungal activities of either the wild type strain MS14 or the mutant MS14GG78 (data not shown). These results further confirmed the essential role of *occE* in the production of antifungal activity of strain MS14.

Mutagenesis of *occT* and *ORF15* did not eliminate the antifungal function of strain MS14

The *occT* mutant MS14SL16 and *ORF15* mutant MS14GG82 were generated by site directed mutagenesis. As compared to the wild type strain MS14 (inhibitory zone radius ± SEM: 13.00 ± 0.58 mm), the antifungal activities of the two mutants were significantly reduced, but not eliminated (MS14SL16: 8.24 ± 0.64 mm; MS14GG82: 8.79 ± 0.38 mm) (Fig. 4.4).

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The putative protein encoded by the *occT* gene carries an ABC transmembrane domain at N terminus and a ATP-binding cassette domain at C terminus, and belongs to bacterial ABC transporter protein family (11). The mutation of the *nptII* insertion in *occT* could not eliminate antifungal activity, indicating the gene may not be absolutely required for secretion of occidiofungin. Disruption of *occT* decreased the secretion amount of occidiofungin, but it might be exported via other pathways. In addition, ABC transporters are conserved and common enzymes in bacteria, it was reported that there are 338 ABC system-associated ORFs in *B. pseudomallei* strain K96243 and 275 ORFs in *B. mallei* strain ATCC 23344 (13). It is hypothesized that a homolog(s) also exist in the strain MS14 genomes, which complement the role of the *occT* gene in export of occidiofungin in the mutant. For example, BLAST search found that there was another homolog of *occT* (bam_1536, amino acid identity: 39%) in chromosome 1 of strain AMMD, in addition to the homolog bam_6469 in chromosome 3.

The putative protein encoded by the *ORF15* gene carries a Te domain, which was predicted to be involved in cyclization and termination of oligopeptide produced by NRPS. However, like *occT*, the mutation in *ORF15* did not eliminate antifungal activity of strain MS14. It is hypothesized that another Te domain, which located at the C terminal of *occA* complemented the role of *ORF15*.

Transcription of all the seven ORFs identified in the region except *ORF16* was regulated by both *ambR1* and *ambR2*, the LuxR-type regulatory genes

To test the relationship between the newly identified genes or ORFs with the LuxR regulatory genes *ambR1* and *ambR2*, Q-PCR analyses were performed with RNAs isolated from strain MS14 and the mutants MS14GG44 and MS14MT15 (Table 4.1). Previous studies demonstrated that both *ambR1* and *ambR2* positively regulated

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transcription of *ORFs 2-9* (12). As expected, significant differences ($P < 0.05$) of expression levels of *ORFs 10-15* in the mutants MS14GG44 and MS14MT15 were observed as compared with those in the wild-type strain MS14. Transcript levels of the ORFs in mutants MS14GG44 and MS14MT15 were reduced 13.2 and 12.0 folds on average, respectively (Fig. 4.3). These data suggest that transcription of *ORFs 10-15* are promoted by the *ambR1* and *ambR2* gene products.

Transcription of *ORF16* was not significantly affected by mutations in either *ambR1* or *ambR2*, indicating that the *ORF16* is not regulated by either of them. In addition, the ORF16 gene, which codes for a hypothetically conserved protein, has highest identity (93%) to ORF number of the of *B. lata* strain 383 genome ; however, no significant homolog was found in the genome of strain AMMD, which has an uncharacterized genomic region sharing the highest similarity to the *occ* gene cluster (*ambR1* - *ORF15*). These data implies that *ORF16*, similar to *ORF1* (12), may not be part of the gene cluster required for the antifungal activity of strain MS14, and *ORF15* is the last gene at the right border of the gene cluster. These results indicate that the whole length of the *occ* gene cluster, which is responsible for the production of occidiofungin, has been identified and it ranges from *ambR1* to *ORF15* (Fig. 4.1).

Conclusions

The right border of the *occ* gene cluster, required for the antifungal activity of strain MS14, was sequenced and another seven putative genes were identified. Site-directed mutagenesis revealed that *occE* (*ORF11*) was required for production of the antifungal activity against the indicator fungus *G. candidum*. By contrast, *ORF2* and *ORF15* were not absolutely required for the antifungal activity of MS14. Transcription

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of the six putative genes (*ORFs 10-15*) identified in the region except *ORF16* was regulated by the LuxR-type regulatory genes, *ambR1* and *ambR2*. The whole length of the *occ* gene cluster has been characterized, which is composed of 16 ORFs. Among the 16 members of this cluster, ORF5, 6, 7, 9 and 11 (OccA - OccE), which were predicted to be NRPS or NRPS-PKS, are directly relative to the biosynthesis of the antifungal compound occidiofungin. AmbR1 and AmbR2 are the regulators controlling the transcription and expression of occidiofungin. ORF2 (OccT) is predicted to be relative to the secretion of the antifungal compound. ORF4, 12, 13, 14 and 15 were predicted to be involved in the modification of occidiofungin or its components. In addition, sequence analysis indicated that occidiofungin produced by the *occ* gene cluster of strain MS14 is not involved in the potential human pathogenesis. The genetic information of the *occ* gene cluster has provided a solid foundation to enhance production of occidiofungin and to optimize its chemical structure to increase its antifungal activity and to minimize possible toxicity to plants and animals. For example, overexpression of *occT* and/or *ambR1* may increase occidiofungin production by strain MS14.

The study of hybrid NRP-PK natural products, which further expands the perspective of combinatorial biosynthesis, is attracting more attentions recently (9). Some hybrid products with medicinal importance have been reported, such as rapamycin, bleomycin and leinamycin (8,30,32). Understanding the genomic structures for the production of occidiofungin, especially the biosynthesis of the novel amino acid will benefit the optimization and production of novel “unnatural” natural products.

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Table 4.1 Bacterial strains and plasmids

Strains or plasmids	**Relevant characteristics**	**Source**
Escherichia coli		
JM109	*recA1*, *endA1*, *gyr*A96, *thi*, hsdR17, *sup*E44, *rel*A1, Δ(lac-proAB)/F' [traD36, proAB+, *lacIq*, *lacZ*ΔM15]	Promega
Burkholderia contaminans		
MS14	Wild type strain	(11)
MS14MT15	*ambR2*::Tn*5* derivative of MS14; Km^r	(11)
MS14GG44	*ambR1*::*nptII* derivative of MS14; Km^r	(12)
MS14MT24	*ORF9*::Tn*5* derivative of MS14; Km^r	This study
MS14GG78	*occE*::*nptII* derivative of MS14; Km^r	This study
MS14GG82	*ORF15*::*nptII* derivative of MS14; Km^r	This study
MS14SL16	*occT*::*nptII* derivative of MS14; Km^r	This study
Plasmid /fosmid		
pSL604	EZ-Tn*5* carrying the ~1 kb genomic DNA of MS14MT24; Km^r	This study
pBluescript II SK	Cloning vector; Ap^r	Strategene
pBR325	Cloning vector; Cm^r, Tc^r, Ap^r	(25)
pBR325[R1]	pBR325 without *Eco*RI site; Cm^r, Tc^r, Ap^r	(25)
pMLS7	Expression vector of *Burkholderia*; Tp^r	(18)
pGEM-T Easy	Cloning vector; Ap^r	Promega
pBSL15	Kanamycin resistance gene cassette; Km^r	(1)
pBSL15[R1]	Kanamycin resistance gene cassette lacking *Eco*RI sites; Km^r	This study

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Table 4.1 (continued)

pGG15	pGEM-T Easy carrying 1-kb PCR product containing the *ORF15* gene; Ap^r	This study
pGG16	pGG15 with the *nptII* insertion in *ORF15* at the *Sma*I site; Km^r, Ap^r	This study
pGG17	pBR325 carrying the 2.1-kb *Eco*RI fragment containing *ORF15* disrupted by *nptII*; Cm^r, Tc^r, Km^r	This study
pGG20	pGEM-T Easy carrying 4-kb PCR product containing the *occE* gene; Ap^r	This study
pGG21	pGG20 with the *nptII* insertion in *occE* at the *Bam*HI site; Km^r, Ap^r	This study
pGG22	pBR325 carrying the 5-kb *Eco*RI fragment containing *occE* gene disrupted by *nptII*; Cm^r, Tc^r, Km^r	This study
pGG23	pGEM-T Easy carrying 4.5-kb PCR product containing the intact *occE* gene; Ap^r	This study
pGG24	pMLS7 carrying 4.5-kb *Hind*III fragment harboring the intact *occE* gene; Tp^r	This study
pGG25	pBR325[R1] carrying the 2.1-kb HindIII - *Eco*RV PCR fragment containing *occT* gene; Cm^r, Tc^r	This study
pGG26	pGG25 with the *nptII* insertion in *occT* at *Eco*RI site; Cm^r, Tc^r, Km^r	This study
4G5	pCC1FOS carrying 40-kb genomic DNA of MS14; Cm^r	This study

* Km^r: Kanamycin resistance; Ap^r: Ampicillin resistance; Tp^r: Trimethoprim resistance; Cm^r: Chloramphenicol resistance; Tc^r, Tetracycline resistance

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Table 4.2 The putative genes identified at the right border of *occ* gene cluster

Gene or ORF	**Size (bp)**	**Homologue [a]**	**Identity (%)**	**Predicted Function [b]**
ORF10	3324	bamb_6477	92	Short-chain dehydrogenase
ORF11 (*occE*)	4428	bamb_6478	91	Nonribosomal peptide synthetase
ORF12	987	bamb_6479	91	Taurine catabolism dioxygenase
ORF13	1371	bamb_6480	91	Transaminase
ORF14	951	bamb_6481	94	Epimerase / Dehydratase
ORF15	720	bamb_6482	90	Thioesterase
ORF16	288	C7511	93	Hypothetical protein [c]

a: Homolog to the putative proteins of *Burkholderia ambifaria* AMMD (GenBank: NC_008392); b: Predicted functions are based on annotation of strain AMMD and strain 383; c: homolog to C7511 of *B. lata* 383 (GenBank: NC_007509).

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Table 4.3 Primers used in this study

Primer	Sequence (5'-3')
ORF10F	5'-GAGCGTCTGCAGGTTCGGATAGG
ORF10R	5'-TCTCGGCCTGGATTCGCTGGT
occEF	5'-CTTCCCGGCGCACTTCACAG
occER	5'-ATCGTCGCCGGCCGCAATCA
ORF12F	5'-GGAACAGATGGGCCTGATTGAAG
ORF12R	5'-AGCCTTCTGCGCGGATAACG
ORF13F	5'-CCGCATCACGGCTTCATTGAC
ORF13R	5'-CTCCTTCCCGCGGCTGTTTAC
ORF14F	5'-GCACGATGAAGTTGGACACG
ORF14R	5'-CGCCTGCTACGACGAAGG
ORF15F	5'-GCCATCGTTCGCATTTCGTTTC
ORF15R	5'-CAGGCTGGCGGTGGACATCA
ORF16F	5'-CGAGACCGGCTGGCATGTTCA
ORF16R	5'-CTGCGGGAAGTCGGGGCGTAT
MoccEF	5'-GTCCGGGGCAAACACGAAGTC
MoccER	5'-CTCCTTGGATTACGGGGCAGAC
MORF15F	5'-CGCCACCCGTTACGAGGATTC
MORF15R	5'-ACGCGTCCCCTCTTCCTACG
EoccEF*	5'-CCC<u>AAGCTT</u>ATGCTTCCCGATACA
EoccER*	5'-CCC<u>AAGCTT</u>GCTTCTTGTAGTCAG

* Underlined letters represent restriction endonuclease sites.

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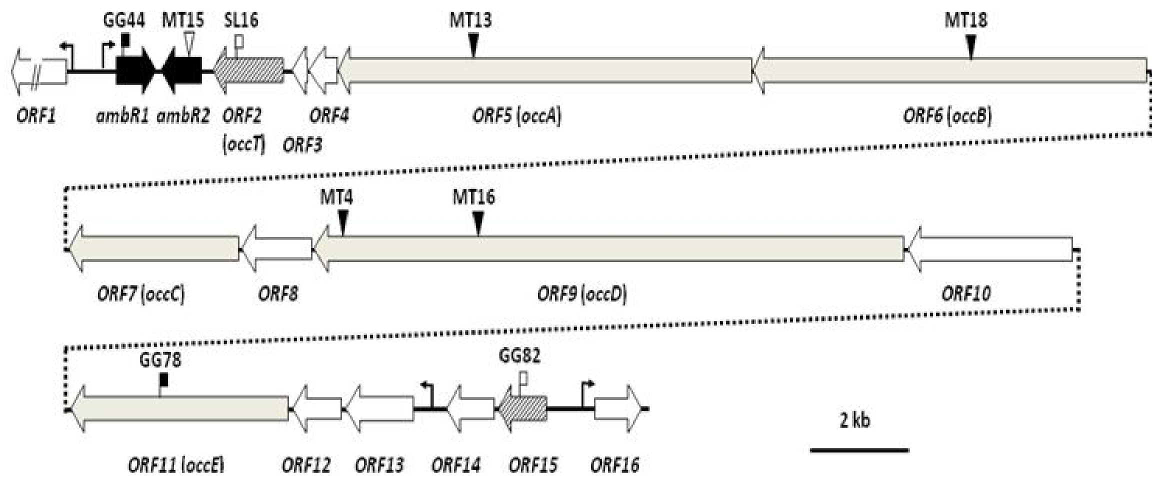

Figure 4.1 Map of 58.2-kb occidiofungin gene cluster of *Burkholderia contaminans* strain MS14.

The positions and orientations of the known genes and potential ORFs are shown as horizontal arrows. Vertical arrows and flags indicate the insertion positions of the Tn*5* transposon and the *nptII* gene cassette, respectively. The open and solid vertical arrows/flags represent reduction and elimination of antifungal activity, respectively. The arrows indicate putative promoter sequence.

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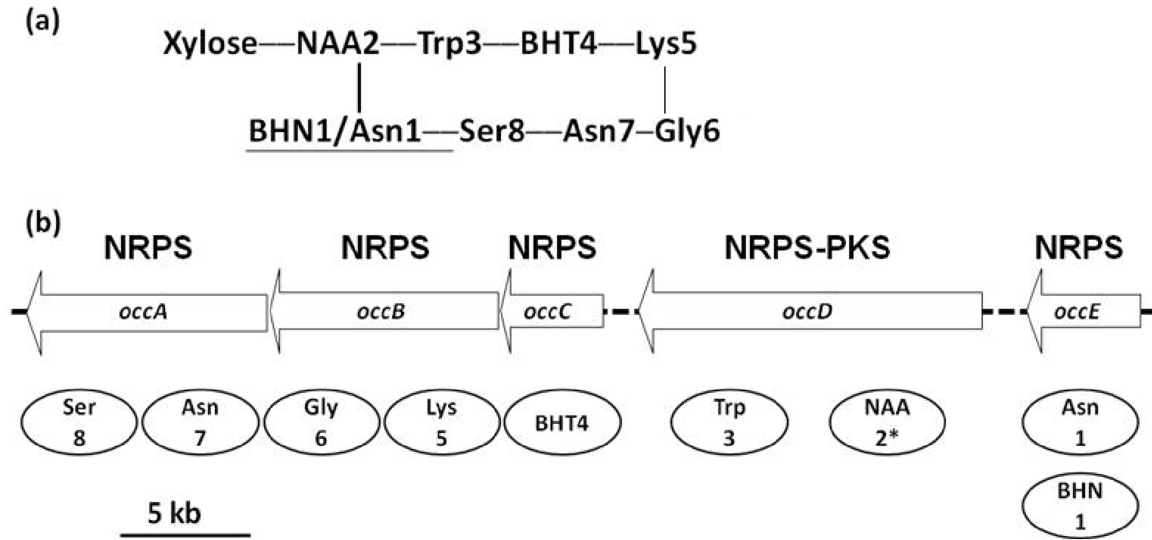

Figure 4.2 Schematic representation of the amino acid modules present in *occ* gene cluster.

Schematic representation of the amino acid modules present in *occA* – *occE*. (a) Primary structure of occidiofungin. BHT, beta hydroxyl tyrosine; BHN, beta hydroxyl asparagines; NAA, Novel amino acid. (b) Modular organization of the *occ* biosynthetic genes. The individual amino acid residues are incorporated by module 1 to module 8 in a collinear manner with the structure of the occidiofungin molecule. NRPS, nonribosomal peptide synthetase; PKS, polyketide synthetase.

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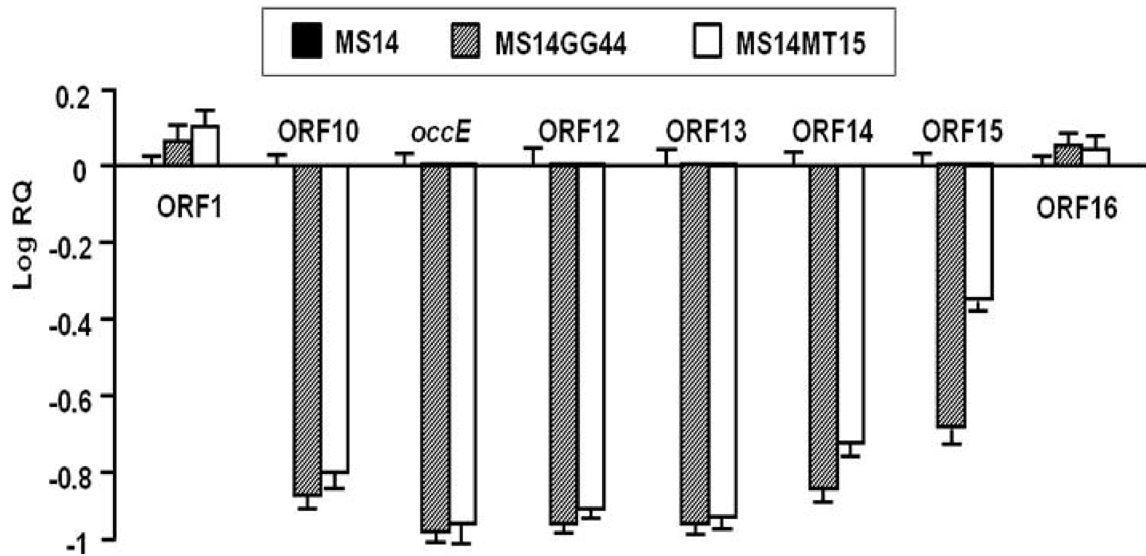

Figure 4.3 Expression of the ORFs identified in the *occ* gene cluster in strain MS14 and its mutants.

Expression of the ORFs identified in the *occ* gene cluster in strain MS14 and its mutants MS14MT15 and MS14GG44 (Table 4.1). Transcript levels of the tested ORFs are presented relative to the transcript levels in the wild type MS14. Mean values for three biological replicates are given, and error bars represent the standard errors of the means. RQ, relative quantification.

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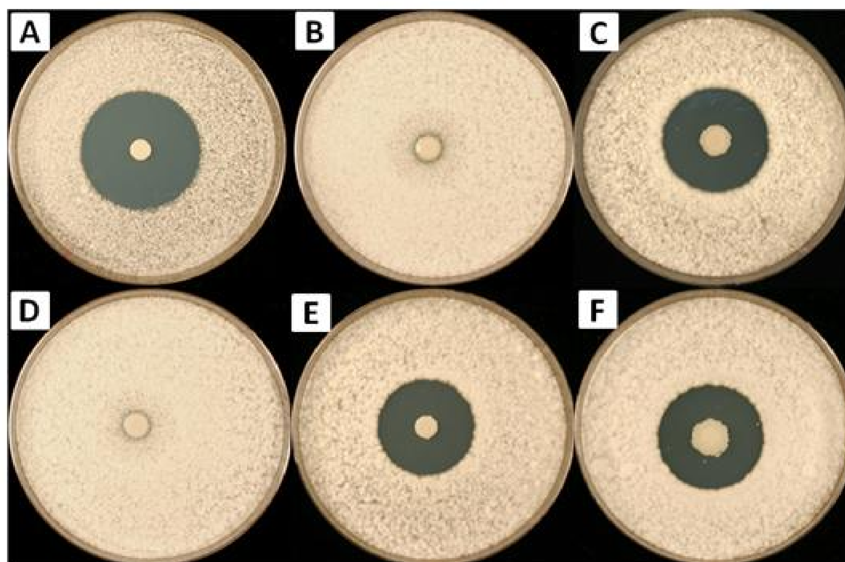

Figure 4.4 Plate bioassays of antifungal activities of *Burkholderia contaminans* strain MS14 with its mutants.

Potato dextrose agar plates were inoculated with each of the strains and incubated for 3 days at 28°C. The plates were oversprayed with the indicator fungus *Geotrichum candidum* and further incubated overnight. A: The wild-type strain MS14; B: MS14GG78; C: MS14GG78 (pGG24); D: MS14MT24; E: MS14GG82; F: MS14SL16.

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