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Research Article

Engineering of LysR-type Regulator DmlR in *Burkholderia ubonensis* CP01 to Enhance Its Antifungal Production against *Ganoderma boninense*

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ABSTRACT

The utilization of an antifungal substance, occidiofungin and burkholdine, derived from *Burkholderia ubonensis* CP01 has displayed promising results in the management of basal stem rot caused by *Ganoderma boninense*. The study aims to further enhance the antifungal production of *B. ubonensis* CP01 through genetic modification. Through comparative genetic analysis, we identified the *dmlR* gene in *B. ubonensis* CP01, which is homologous to the *scmR* gene, a LysR-type transcriptional regulator (LTTR), in *B. thailandensis*. Deleting the *dmlR* gene in CP01 resulted in a complete loss of antifungal synthesis. In contrast, overexpression of this gene led to a substantial increase in antifungal production, as determined by an agar well diffusion assay. These findings suggest that *dmlR* acts as a positive regulator of antifungal gene expression in *B. ubonensis* CP01. RP-HPLC analysis revealed that the mutant strain overexpressing the *dmlR* gene (mutant WB12) produced a higher peak at the 24-25 minute elution time. Previous high-resolution mass spectrometry analysis by our group identified the compound at this peak as six analog compounds with monoisotopic masses similar to those of cyclic lipopeptides, including occidiofungin and burkholdine. The WB12 mutant exhibited approximately 15% higher concentrations of antifungal compounds than the wild type. Additionally, whole genome sequencing confirmed that the introduced *dmlR* gene had been integrated into the locus on chromosome 2 of *B. ubonensis* CP01. LTTRs play a pivotal role in regulating the production of antifungal agents in CP01. Furthermore, it highlights the potential for manipulating LTTRs to enhance the desirable characteristics of the *Burkholderia* genus in regard to the production of secondary metabolites.

1. Introduction

Palm oil is an incredibly efficient crop. According to the Organisation for Economic Co-operation and Development (OECD) and the Food and Agriculture Organization (FAO), OECD-FAO Agricultural Outlook, palm oil accounts for 36% of the global vegetable oil supply. By 2030, Indonesia and Malaysia are projected to account for 82% of global palm oil production and 55% of global vegetable oil exports (OECD/FAO 2024). For the past two decades, *Ganoderma boninense*-induced basal stem rot (BSR) has been recognized as the most significant threat to the sustainable cultivation of oil palms in Southeast Asia. Coupled with the effects of shifting climate patterns, it is anticipated that by 2100, *G. boninense* could have a substantial impact, ranging from 41 to 100% in Sumatra and various regions of Malaysia (Abubakar *et al.* 2022). The threat posed by

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this slow-progressing pathogen is largely due to the lack of effective commercial detection and control methods. Encouragingly, the application of biological agents has shown promising outcomes in restraining the proliferation of *G. boninense*, both in controlled settings and in real-world conditions (Muniroh *et al.* 2019; Keerthana *et al.* 2023).

Some members of the genus *Burkholderia* have been reported to have antagonistic activity against a broad range of plant pathogens. *Burkholderia cepacia* produced secondary metabolites such as pyrrolnitrin, phenazine, cepabactin, and other unidentified volatile or non-volatile compounds (Cartwright *et al.* 1995). *B. contaminans* strain MS14 produces antifungal occidiofungin, responsible for antifungal activities against plant and animal fungal pathogens (Chen *et al.* 2013). In the previous study, we isolated and characterized a potential biocontrol agent for *G. boninense*, namely *Burkholderia ubonensis* CP01 (Prihatna *et al.* 2022). Similar to strain MS14, this bacterium has demonstrated the capacity to generate antifungal compounds, predicted as occidiofungin and burkholdine, at substantial levels within cultures, offering the possibility of formulation as environmentally friendly fungicides.

The secondary metabolites produced by *Burkholderia*, often through nonribosomal peptide synthetases (NRPS) and polyketide synthetases (PKS) gene clusters, comprise a set of genes. Each gene encodes a distinct enzyme domain or functional element vital for the assembly process of the NRPS metabolite (Meng *et al.* 2023; Yang *et al.* 2023). Recent investigations have highlighted the role of LysR-type transcriptional regulators (LTTRs), such as ScmR, as key factors controlling the expression of biosynthetic gene clusters involved in antifungal production (Mao *et al.* 2017; Duong *et al.* 2018; Yu *et al.* 2023). LTTRs constitute a versatile group of transcription factors widely distributed among bacterial species. Characterized by a modular architecture comprising DNA-binding and effector-binding domains, LTTRs demonstrate an extraordinary ability to adapt to a wide spectrum of environmental cues. Through their interactions with operator sequences situated within gene promoters, LTTRs can either activate or suppress transcription, thereby influencing various cellular processes, including metabolism, stress response, and the biosynthesis of secondary metabolites (Baugh *et al.* 2023). Understanding their roles and the underlying molecular mechanisms is essential in formulating strategies to enhance the production of *Burkholderia ubonensis* CP01 antifungal compounds.

In this study, we identified *dmlR* gene in *B. ubonensis* CP01, which is homologous to the conserved *scmR* in the *Burkholderia* genus. Furthermore, overexpression was achieved through random insertion using the Tn5 plasmid to obtain an overproducing mutant, designated WB12. The evidence from this research is hoped to provide additional insight into the prevalence and pivotal role of LTTR as a secondary metabolite regulator, as well as its manipulation for improving beneficial traits in *B. ubonensis* CP01.

2. Materials and Methods

2.1. Bacterial Strains, Fungal Strains, and Culture Conditions

Burkholderia ubonensis CP01 was isolated from the oil palm rhizosphere (Prihatna *et al.* 2022). The bacterium was cultured in King's B agar (King *et al.* 1954) without antibiotics for the wild-type strain, and with trimethoprim (50 µg/mL) for the mutant strain. Plates were incubated at 30°C for 2-3 days. For assessing antifungal activity, strains of *Ganoderma boninense* B29 (Purnamasari *et al.* 2012) and *Saccharomyces cerevisiae* were employed. *S. cerevisiae* was used as a model because it was easier and faster to use than *G. boninense* to screen for *B. ubonenesis* CP01 mutants. *G. boninense* and *S. cerevisiae* were cultured in potato dextrose agar (PDA, Difco, Sweden) at 30°C for 7 days and 2 days, respectively. *E. coli* TOP 10, *E. coli* DH5α, *E. coli* HB101 and *E. coli* S17 λ-pir were cultured in Luria agar (LA) medium (tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L, and agar 15 g/L) at 37°C for one day.

2.2. *dmlR* gene Knockout through Homologous Recombination

Genomic DNA was isolated from *Burkholderia ubonensis* CP01. The *scmR* gene was targeted following the approach outlined by Mao *et al.* (2017). A Clustal Omega 1.2.4 alignment (Sievers *et al.* 2011) was performed to assess the similarity of the protein sequences of this gene among *B. pseudomallei* Bp82, *B. mallei* GB8, and *B. thailandensis* E264 as described by *Mao et al.* (2017). The author examined RNA-seq datasets to identify transcriptional regulators conserved across these three strains. Subsequent investigations to find homologs in *B. ubonensis* CP01 using BLAST (Altschul *et al.* 1997) revealed that the *dmlR* gene exhibited the highest level of homology. Conserved domain analysis using the NCBI Conserved Domain

Database (CDD) (Wang *et al.* 2023) was conducted to assess the upstream region of dmlR. Knockout mutant bacteria of *B. ubonensis* CP01 were generated through homologous recombination using primers listed in Table 1. DNA oligomers were purchased from Macrogen (Seoul, South Korea). The *dmlR* gene was amplified using Phusion High-Fidelity DNA Polymerase (NEB, USA) according to the provided guidelines. The pUC19 plasmid (Pharmacia P-L Biochemicals, USA) served as the construction platform for integrating the *dmlR* gene. The resultant amplicon and pUC19 backbone vector were subjected to overnight digestion with the restriction enzymes *Hind*III and *BamH*I. Calf intestinal phosphatase (CIP) was introduced as per the NEB guidelines to avoid self-ligation of the plasmid. The digested amplicon and pUC19 were then purified using the QIAquick PCR purification kit (Qiagen, Darmstadt, Germany) to remove restriction enzymes and ensure successful cloning. Following an overnight ligation using T4 DNA ligase (NEB, USA), the combined sample was introduced into *E. coli* TOP 10 competent cells via the heat shock technique. The resulting colonies, which emerged on LA medium infused with ampicillin (100 µg/mL), were PCR-amplified and sequenced using M13F and M13R primers (Table 1), ensuring the correct insertion of the *dmlR* gene into the pUC19 plasmid.

A trimethoprim-resistant gene (*dfrB2*) was added to the cloning vector due to the multiple resistance features of *B. ubonensis* CP01. The *dfrB2* gene was extracted from the modified pBluescript plasmid (WBE culture collection) via amplification with the Phusion® High-Fidelity DNA Polymerase (NEB, USA) using primers listed in Table 1 and underwent overnight digestion with *Pst*I as per Promega's instructions. Concurrently, the pUC19 containing the dmlR gene was also digested overnight with PstI (Promega, USA). After an overnight ligation, the combined sample was reintroduced into *E. coli* TOP 10 competent cells via the heat shock method. The pUC19_*dmlR_dfrB2* plasmid was further amplified to extract the *dmlR_dfrB2* region, which was subsequently purified using the QIAquick® Gel Extraction Kit (Qiagen, Germany). This amplicon underwent phosphorylation using the T4 PNK Enzyme (NEB, USA).

In parallel, the pUT-mini-Tn5 plasmid (de Lorenzo *et al.* 1990) was employed as a suicide vector for homologous recombination. After another overnight ligation of *dmlR_dfrB2* with pUT mini using the T4 DNA ligase (NEB, USA), the ligation mixture was introduced into *E. coli* S17 λ-pir competent cells via heat shock. Subsequently, the pUT mini carrying the *dmlR_dfrB2* gene, pUT-mini-Tn5_*dmlR_dfrB2*, was transformed into *B. ubonensis* CP01 competent cells via electroporation with the Bio-Rad Gene Pulser Electroporation Systems (BioRad, USA). Colonies were selected on LA medium supplemented with ampicillin (100 µg/mL) and trimethoprim (50 µg/mL). To ensure the accuracy of each transformation step, the plasmids retrieved were sequence-verified and digested with the appropriate restriction enzymes.

2.3. Complementation Study to Restore *dmlr* Gene Regulator Function

The construction of the plasmid included the promoter, coding sequence (CDS), and stop codon of the *dmlR* gene. The native promoter of the *dmlR* gene, 345 bp in length, was used and positioned upstream of the gene. It was predicted using the SAPPHIRE online tool (Coppens and Lavigne 2020). The promoter and CDS region of the *dmlR* gene were amplified using CP01_pro-*dmlR*_F_01_*Xba*I and CP01_*dmlR*_R_*Hind*III (Table 2). The dmlR full gene was amplified using Phusion High-Fidelity DNA Polymerase (NEB, USA) according to the manufacturer's instructions. pRK415:Tcr was used as a construction plasmid for the *dmlR* full gene. The amplicon and backbone vector pRK415 were digested overnight with *Xba*I and *Hind*III (NEB, USA) and then ligated overnight using T4 DNA ligase (NEB, USA). The plasmids of pRK415_pro-*dmlR_Xba*I-*Hind*III were then transformed into competent cells of *E. coli* DH5α. For transformation via conjugation, helper *E. coli* HB101 harboring pRK2013:Kanr was used, as this is a helper plasmid for mobilizing non-self-transmissible plasmids. *E. coli* DH5α was cultured in LA medium, HB101 was

Table 1. List of primers used for manipulation of the *dmlR* gene in *B. ubonenesis* CP01

Primer	Sequence	Amplicon size (bp)
CP01_*dmlR*_F_*Hind*III	TATCAAGCTTAACCAGATTCAGACCATGCGT	1,019
CP01_*dmlR*_R_*BamH*I	TATCGGATCCTGGGAGGCGCAGTTTACTTTA	
dfrB2_Prom_PstI	TATCCTGCAGTCGACATAAGCCTGTTCGGT	611
dfrB2_3'_PstI	TATCCTGCAGTTAGGCCACACGTTCAAGTGC	
M13F	GTAAAACGACGGCCAG	
M13R	CAGGAAACAGCTATGAC	

Table 2. List of primers used for the complementation study of mutant ΔdmlR and overexpression of *dmlR*

Primer	Sequence	Amplicon size (bp)
CP01_pro-*dmlR*_F_01_*Xba*I	TACGTTCTAGAAGGGAATCGGCGAGTAATCG	1,823
CP01_*dmlR*_R_*Hind*III	TACGTAAGCTTTGGGAGGCGCAGTTTACTTTA	

cultured in LA medium supplemented with kanamycin (25 µg/mL) at 37°C for 1 day, while *B. ubonensis* CP01 mutant Δ*dmlR* was grown in King's B medium supplemented with trimethoprim (50 µg/mL) at 30°C for 2-3 days.

Bacteria were transformed via triparental mating involving three bacteria, which are helper bacteria (H), donor bacteria (D), and recipient bacteria (R). In this study, H, D, and R were *E. coli* HB101:pRK2013, *E.coli* DH5α:pRK415:*dmlR* with the promoter, and *B. ubonensis* CP01 mutant Δ*dmlR*, respectively. R, D, and H were incubated overnight at 37°C for *E. coli* and 30°C for *B. ubonensis* CP01. A 1.5 mL overnight culture was centrifuged at 6,000 rpm for 1 minute, followed by removal of the supernatant. The pellet was washed with 1 mL of Luria broth (LB) twice. The remaining supernatant was mixed with 200 µL of LB for mating. Strains R, D, and H were mixed at a 3:1:1 ratio to create the experimental condition. Negative controls included pairwise combinations (R+D, D+H, and R+H) mixed at a 1:1 ratio, as well as individual cultures of R, D, and H plated separately. For each condition, 30–50 µL of culture was applied to sterile 0.45 µm filter paper placed on LA medium and incubated at 30°C for 6 hours. Following incubation, the filter papers were transferred into 500 µL of 0.85% NaCl solution and vortexed thoroughly. From each control mixture, 50 µL was plated onto counter-selection media containing antibiotics. For the R+D+H mixture, the suspension was first serially diluted up to 10^6, and 50 µL, 100 µL, and 200 µL aliquots were plated onto counter-selection media. All plates were incubated at 30°C for 3–5 days.

2.4. Overexpression of *dmlR*

The *DmlR* full gene, obtained from the previous amplification (up to 300 pmol of 5' termini of *DmlR* gene) with primer pairs listed in Table 2, underwent phosphorylation using the T4 PNK Enzyme (NEB, USA). The pUT-mini-Tn5:Sp^r/Sm^r:Tp^r:Amp^r plasmid was subjected to overnight digestion with BstEII following NEB instructions. Given that the amplicon had a blunt end and the plasmid had a sticky end, a blunting reaction was performed using T4 DNA polymerase according to the NEB protocol. Both the amplicon and plasmid were subsequently purified using the QIAquick PCR purification kit (Qiagen, Germany). An overnight ligation using the T4 DNA ligase (NEB, USA) was performed, and the resultant mixture was introduced into *E. coli* S17 λ-pir competent cells through the heat shock method. Colonies that grew in a selective medium, supplemented with ampicillin (100 µg/mL) and trimethoprim (50 µg/mL), were amplified using a PCR and sequenced with CP01_pro-dmlR_F_01_XbaI and CP01_dmlR_R_HindIII. The pUT mini harboring the *dmlR* full gene (pUT-mini-Tn5:Sp^r/Sm^r:Tp^r:Amp^r_*dmlR*) in *E. coli* S17 λ-pir was then transferred into *B. ubonensis* CP01 competent cells via bi-parental mating. Transconjugation frequency was calculated as (total transconjugant cells/mL) ÷ (total recipient cells/mL) × 100%. An overnight culture (1.5 mL) was centrifuged at 6,000 rpm for 1 minute, and the supernatant was discarded. The resulting cell pellet was washed twice with 1 mL of LB medium to remove residual components. The final pellet was then resuspended in 200 µL of LB medium and used for the mating procedure. The recipient (R; CP01) and donor (D; *E. coli* S17 λ-pir) were mixed at a ratio of 1:1 or 7:3 to increase the mating efficiency. A mixture of 30-50 µl of R+D, R, and D cultures was placed on a 0.45 µm sterilized filter paper in LA medium. The plates were then incubated for 6 hours at 30°C, following our laboratory-optimized mating procedure (unpublished result). The filter paper was placed in 500 µL of 0.85% NaCl solution and vortexed after incubation. For the counter selection, 50 µl of each negative control and 50 µL, 100 µL, and 200 µL of the R+D mixture were plated onto media with antibiotics. All plates were subsequently incubated at 30°C for 3 to 5 days. A list of all genes and plasmids used in this study is shown in Table 3.

2.5. Antifungal Activity Assay

The antifungal activity of the *B. ubonensis* CP01 mutant was evaluated using an agar well diffusion assay with adjustments, following the protocol by Katoch and Pull (2017). An overnight culture was subjected to centrifugation at 10,000×g for 2 minutes, and the resulting supernatant was decanted. The supernatant was then subjected to a 10-minute boiling period at 85°C to eliminate any remaining live cells. According to Prihatna *et al.* (2022), strain CP01 produced a small 1.2-kDa antifungal peptide (AFP), likely a cyclic peptide, which

Table 3. List of genes and plasmids used and designed in this study

Name	Type	Description	Usage
dmlR	Gene	LysR-type transcriptional regulator in *B. ubonensis* CP01	The whole study
dfrB2	Gene	Trimethoprim resistance gene	Knockout study
dmlR_dfrB2	Gene	*dmlR* gene disrupted with the trimethoprim resistance gene in between	Knockout study
pUC 19	Backbone plasmid	Construction plasmid for integrating the *dmlR* and *dfrB2* genes	Knockout study
pBluescript	Backbone plasmid	Plasmid for the extraction of the *dfrB2* gene	Knockout study
pUT mini Tn5	Backbone plasmid	A suicide vector for homologous recombination	Knockout and overexpression study
pUC19_*dmlR*	Recombinant plasmid	pUC19 carried *dmlR* gene	Knockout study
pUC 19_*dmlR_dfrB2*	Recombinant plasmid	pUC19 carried the *dmlR* gene and the trimethoprim resistance gene	Knockout study
pUT-mini-Tn5_*dmlR_dfrB2*	Recombinant plasmid	pUT mini carried the *dmlR* gene and the trimethoprim resistance gene for homologous recombination	Knockout study
pRK415:Tc^r	Backbone plasmid	pRK415 contained a tetracycline resistance gene	Complementation study
pRK2013:Kan^r	Backbone plasmid	Helper plasmid for triparental mating	Complementation study
pRK415_pro-*dmlR_Xba*I-*Hind*III	Recombinant plasmid	pRK415 carried the *dmlR* full gene	Complementation study
pUT-mini-Tn5:Sp^r/Sm^r:Tp^r:Amp^r	Recombinant plasmid	The modified pUT mini carried ampicillin, streptomycin, spectinomycin, and trimethoprim resistance genes	Overexpression study
pUT-mini-Tn5:Sp^r/Sm^r:Tp^r:Amp^r_*dmlR*	Recombinant plasmid	The modified pUT mini carried the respective antibiotic resistance gene and *dmlR* full gene	Overexpression study

is generally stable against heat and protease hydrolysis. The boiling step was intended to inactivate the bacteria, as handling live cells presents greater complexity and contamination risks. Since the antifungal compound is secreted extracellularly, the inactivation of the cells does not interfere with its activity. Therefore, boiling the supernatant is not expected to denature or reduce the efficacy of the antifungal peptide.

Roughly 100 µL of the boiled supernatant was carefully added to a well created by piercing a pipette tip into PDA medium that had been previously inoculated with S. cerevisiae (OD_{600}:0.5) or *G. boninense* agar plug (7 days of incubation). After 48 hours of incubation at 30°C, the radius of the growth inhibition zone was measured. Each experiment was repeated in three replications. Jamovi was used for data representation and statistical analysis with R (R Core Team 2021; jamovi 2022). All data were statistically analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test to identify significant differences between groups, assuming equal variances. A p-value of less than 0.05 was considered statistically significant. It is important to note that no normality test was performed in this study.

2.6. Antifungal Compound Analysis using RP-HPLC

The targeted secondary metabolites, occidiofungin and burkholdine, from both the *B. ubonensis* CP01 wild-type and its mutant strain WB12 were identified using reversed-phase high-performance liquid chromatography (RP-HPLC) following the method previously described by Prihatna *et al.* (2022). The cell-free culture supernatant was obtained by centrifuging a 100 mL overnight culture (OD_{600} = 2.0) grown in King's B medium at 10,000 × g for 15 minutes. To precipitate the antifungal compounds, ammonium sulfate was added to the cell-free culture, reaching a concentration of 50% (w/v), followed by stirring on ice for 1 hour. The mixture was then subjected to centrifugation at 10,000 g for 15 minutes. The resulting pellet, containing the antifungal compounds, was collected and resuspended in 5 ml of 35% (v/v) acetonitrile (Merck, Darmstadt, Germany), thereby concentrating the antifungal extract 20 × (from 100 ml of culture to 5 ml of acetonitrile). The suspension was syringe-filtered with a 0.2-µm nylon filter (Millex Syringe Filters; Millipore). The filtrates were injected into an analytical HPLC system equipped with a ZORBAX 300SB C-18 column (Agilent; Santa

Clara, CA) at 30°C, connected to an Agilent 1200 Series chromatograph (Agilent, Germany) and a diode array detector with detection at a wavelength of 220 nm. HPLC analysis was performed using a gradient of 0.1% (v/v) trifluoroacetic acid (Sigma-Aldrich, St. Louis, MO) and acetonitrile (9:1) at a flow rate of 0.5 mL/min. The gradient was as follows: 10% acetonitrile for 1.6 times the column volume, 10 to 90% acetonitrile for 5.4 times the column volume, and 10% acetonitrile for 2 times the column volume. The total run time was 45 minutes. A fraction collector was employed to collect fractions based on the ascending (5 AU/min) and descending slope (10 AU/min) of the peaks. The mobile phase of the collected fractions was evaporated using a vacuum evaporator, leaving behind a solid esidue that was subsequently resuspended in 35% (v/v) acetonitrile. These collected fractions were then assessed for their antifungal activity against *S. cerevisiae*. Following a 48-hour incubation period at 30°C, the radius of the growth inhibition zone was measured.

2.7. Identification of the Gene Affected by Tn5 Insertion

The Wizard Genomic DNA Purification Kit (Promega, USA) was used to purify genomic DNA from overnight LB cultures, which were 0.5–1 mL in volume. The concentration and purity of the DNA were determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA). DNA quality control and whole-genome sequencing of mutant WB12 were performed at Genetika Science Indonesia using the GridION sequencer (Oxford Nanopore Technologies), operated with MinKNOW software version 20.06.9. Base-calling of the raw signal data was performed using Guppy version 4.0.11 in high-accuracy mode (Wick *et al.* 2019). This version was used instead of a newer release because the analysis was conducted in 2022. All generated FASTQ files were then filtered using Filtlong v0.2.1 software (Wick 2023), with a minimum read length threshold of 1 kb (--min_length 1000) and a selection of the highest-quality reads until a total of 500 Mb of bases was retained (--target_bases 500000000). The quality of the filtered reads was then assessed using the NanoPlot v1.40.0 visualization tool (De Coster *et al.* 2018). De novo reads assembly was conducted using Flye software version 2.9.1 (Kolmogorov *et al.* 2019) followed by assembly polishing using Medaka v1.7.0 software (Oxford Nanopore Technologies Research 2023). Annotation was conducted using the NCBI Prokaryotic Genome Annotation Pipeline (Tatusova *et al.* 2016). The assembled genomes and their annotation were assessed using Benchmarking Universal Single-Copy Orthologs v5 (BUSCO) software (Simão *et al.* 2015). The analysis was conducted against the Burkholderiales_odb10 dataset, resulting in an overall completeness score of 98.7%. Specifically, 98.3% of the BUSCOs were identified as complete and single-copy, 0.4% as complete and duplicated, 0.4% as fragmented, and 0.9% were missing.

3. Results

3.1. Identification of the *scmR* Homologous Gene in *Burkholderia ubonensis* CP01

To identify a homologous gene of *scmR* in *Burkholderia ubonensis* CP01, a search for similarities was carried out using gene BTH_I1403 from *B. thailandensis* E264, BP1026B_I0582 from *B. pseudomallei* Bp82, and BMA2049 from *B. mallei* GB8 obtained from NCBI. A Clustal Omega 1.2.4 alignment of the corresponding protein sequences: *B. pseudomallei* Bp82 (AFI65246.1), *B. mallei* GB8 (AAU49591.1), and *B. thailandensis* E264 (WP_009889425.1), revealed 100% identity between *B. pseudomallei* and *B. mallei*, and 98.77% identity between these two and *B. thailandensis*. The *B. thailandensis* E264 protein sequence was used as a query to identify homologs in *B. ubonensis*. A BLASTP search against the NCBI with standard database revealed a LysR family transcriptional regulator in *B. ubonensis* with 70.64% similarity, 100% query coverage, and an E-value of 8e-166. A local BLAST search against the *B. ubonensis* genome identified *dmlR* as the most similar gene, with 75.5% sequence similarity, 87.6% query coverage, and an E-value of 0. Conserved domain analysis using the NCBI CDD showed that the upstream region of *dmlR* aligns with the bacterial regulatory helix-turn-helix domain typical of the LysR family (E-value: 3.42e-17). The remaining part of the sequence corresponds to the C-terminal substrate-binding domain found in LysR-type transcriptional regulators CrgA, which contains a type 2 periplasmic binding domain (E-value: 4.16e-59).

The promoter of this gene was identified to be located upstream, with a -10 sequence of TTGCCA and a -35 sequence of CATGTT. The gene has an open reading frame of 984 bp and encodes a putative 327 amino acids. This gene was then annotated as belonging to the LysR-type transcriptional regulator (LTTR) family.

3.2. *dmlR* Acts as a Positive Regulator of Antifungal Production in *Burkholderia ubonensis* CP01

To investigate the regulatory role of DmlR in the regulation of antifungal production, we generated a *dmlR* mutant using homologous recombination. Initially, we conducted PCR amplification to create a truncated 1,019 bp segment of the *dmlR* gene, which was subsequently cloned into the plasmid pUC19. This process yielded the recombinant plasmid pUC19_*dmlR*. The trimethoprim resistance gene *dfrB2* (611 bp) was incorporated into the *dmlR* gene. Thus, the *dmlR* sequence was split into ±500 bp sections on both sides. The final construct, depicted in Figure 1A, was introduced into *E. coli* S17 λ-pir through electroporation with electric capacity of 25 µF, 200 Ohm resistance, and 2.5 kV of electric potential for 5 ms. Cells were recovered and transferred into new 1.5 mL tubes, and then incubated at 30°C for recovery. Intergeneric conjugation between *E. coli* S17 λ-pir and *B. ubonensis* CP01 yielded three exconjugants that exhibited resistance to both ampicillin and trimethoprim, conferred by the plasmid. Following confirmation of gene inactivation, the positive exconjugants were cultivated on a PDA medium inoculated with *Ganoderma boninense* B29 for antifungal analysis. The results showed that the *s* mutants exhibited no antifungal activity against *G. boninense*, implying that DmlR functions as a positive regulator of antifungal activity in *B. ubonensis* CP01 (Figure 1B).

3.3. Complementation Study to Restore DmlR Regulator Function

The final construct of DmlR with its native promoter in the pRK415 vector resulted in a plasmid of 12.4 kbp in size (Figure 2), which conferred tetracycline resistance. Since the recipient strain, *B. ubonensis* CP01 Δ*dmlR*, used in this study already contains a trimethoprim resistance gene, the same antibiotic could not be used as a marker. Moreover, as we previously observed, *B. ubonensis* CP01 is resistant to nearly all commonly used antibiotics in our laboratory (Table 4), making transformation selection particularly challenging. To address this, we chose tetracycline as the antibiotic marker and increased its concentration in the medium. A tetracycline concentration of 200 µg/mL (20 times the usual concentration) was anticipated to inhibit the growth of *B. ubonensis* CP01 sufficiently. Triparental mating was performed using a R:D:H ratio of 3:1:1. The proportion of the recipient was increased with the assumption that *B. ubonensis* CP01 growth might be influenced by quorum sensing. Therefore, a higher concentration of the recipient was expected to promote the overgrowth of exconjugants of CP01. Additionally, a tetracycline concentration of 200 µg/mL might no longer be able to inhibit the growth of the exconjugant of *B. ubonensis* CP01 after mating, since tetracycline is not highly effective against the wild-type *B. ubonensis* CP01. No colony was observed in any of the negative controls (Table 5), indicating that no spontaneous mutation or contamination had occurred. Thus, this made the transconjugant selection easier and more reliable.

A total of 60 colonies were selected and tested for antifungal activity in PDA spread with yeast, and then incubated for 2 days. Four out of 60 colonies, namely colonies 10, 32, 49, and 50, showed small clear zones on PDA yeast assayed with the streaking method (Figure 3A and B), as well as with antifungal extraction on 50 ml KB supplemented with Tetracycline 200 µg/mL (Figure 3C). No clear zone was observed from mutant Δ*dmlR* (mutant M3). Unfortunately, these four mutants were difficult to maintain, as they did not grow when streaked several times on KB agar supplemented with 200 µg/mL tetracycline. This suggests that *B. ubonensis* CP01 has either eliminated or failed to retain the plasmid carrying *Tetr* and *dmlR*, as only heterologous expression occurred without evidence of chromosomal integration (unpublished results). A tetracycline concentration of 200 µg/mL might be too toxic for *B. ubonensis* CP01 to resist after the plasmid carrying Tet^r has been "kicked away". Moreover, no dmlR band with a size of 1,823 bp was observed from the genomic DNA of these mutants.

3.4. Enhanced Antifungal Production through the Overexpression of *dmlR*

Biparental mating was employed using the donor plasmid pUT-mini-Tn5 containing *dmlR* with its native promoter to generate mutants with enhanced antifungal activity (Figure 4A). The insertion of the Tn5 plasmid occurred randomly within the genome. A total of 80 transformant colonies were selected and subjected to testing for antifungal activity on PDA plates coated with *S. cerevisiae*. Among these, one colony exhibited a strikingly larger clear zone compared to the parental wild-type (WT) strain, which was then identified

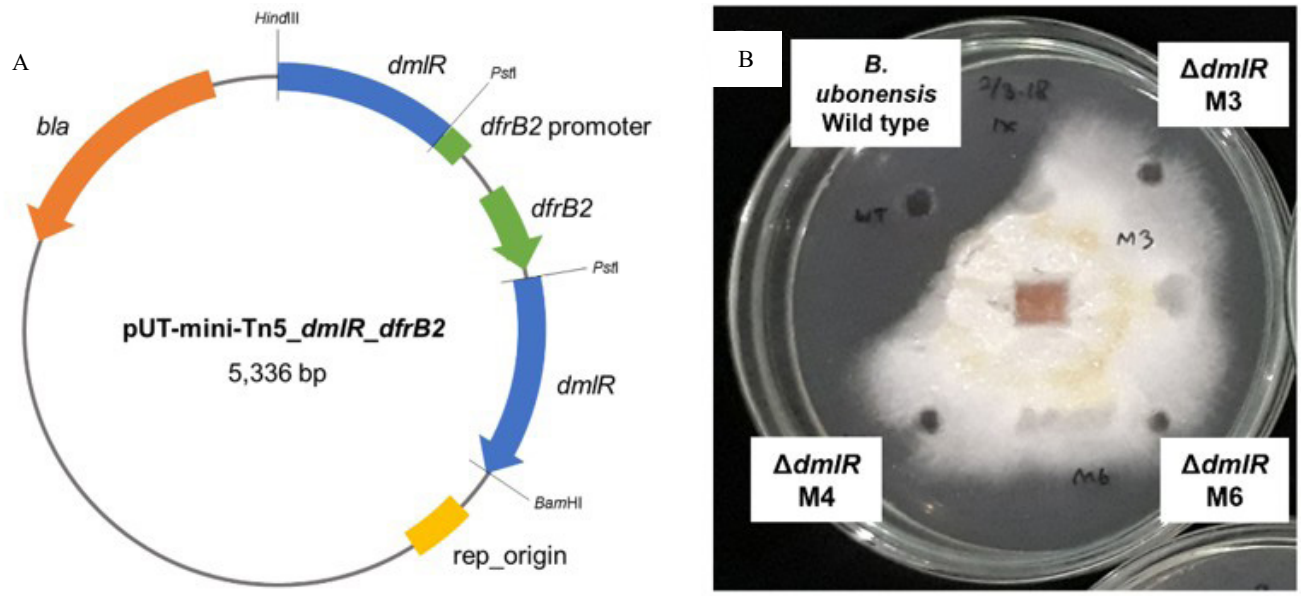

Figure 1. (A) Plasmid construct used for generating Δ*dmlR* strains. The *dfrB2* gene, conferring trimethoprim resistance, was inserted between the *dmlR* gene in the pUT-mini-Tn5 plasmid to facilitate homologous recombination and disrupt the *dmlR* gene, (B) antifungal activity of the *B. ubonensis* wild type and Δ*dmlR* mutants (labeled as M3, M4, and M6) against *Ganoderma boninense*

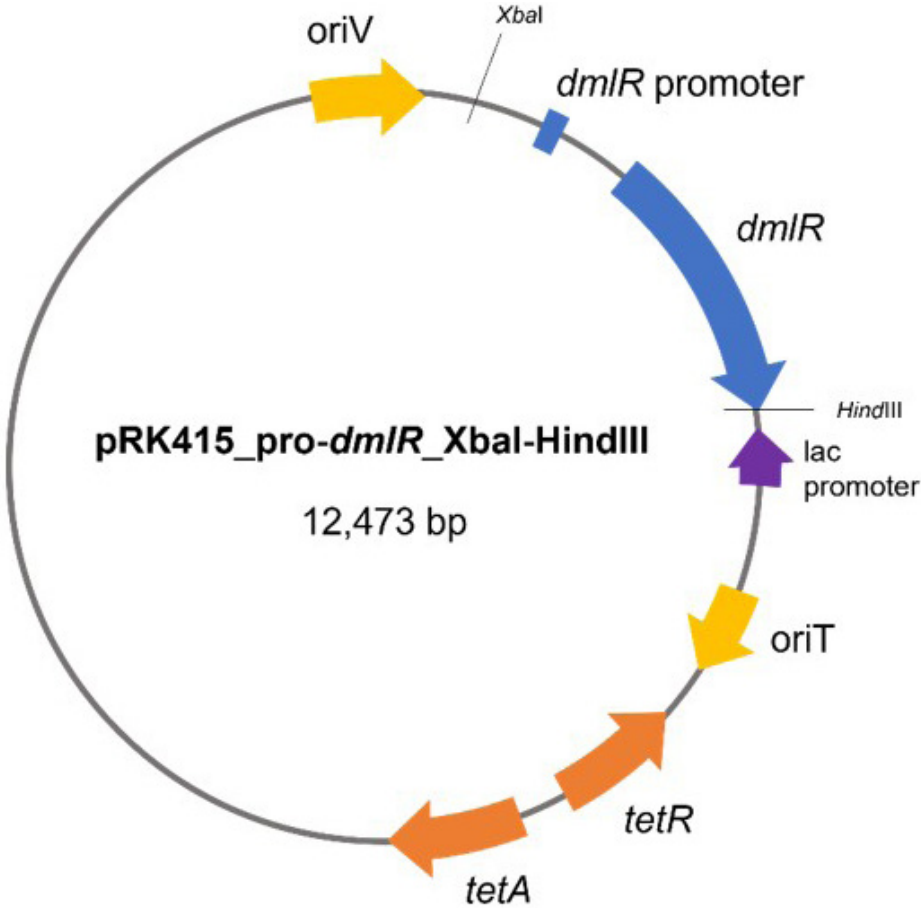

Figure 2. Plasmid constructs for the complementation study. The full *dmlR* gene, including its native promoter, was inserted into the pRK415 vector, which carries the Tetr gene for tetracycline resistance

Table 4. Resistance of *B. ubonensis* CP01 to selected antibiotics grown on King's B medium

Antibiotic	Concentration
Gentamycin	50 µg/mL
Hygromycin	30 µg/mL
Streptomycin	50 µg/mL
Spectinomycin	50 µg/mL
Kanamycin	25 µg/mL
Tetracycline	10 µg/mL
Ampicillin	100 µg/mL
Chlorampenicol	35 µg/mL

Table 5. Summary of negative controls employed for triparental mating in this complementation study

Negative control		Antibiotics used for selection
R+D	Recipient + Donor	Trimethoprim (50 µg/mL) and tetracycline (200 µg/mL)
R+H	Recipient + Helper	Trimethoprim (50 µg/mL), kanamycin (25 µg/mL), and tetracycline (200 µg/mL)
D+H	Donor + Helper	Kanamycin (25 µg/mL), and tetracycline (200 µg/mL)

as WB12 (Figure 4B). Another colony, WB19, also showed a noticeable difference in clear zone formation, though it has not yet been studied in detail. Insertion site analysis revealed that WB12 harbored the *dmlR* gene, which was inserted within a gene encoding a gluconate family permease, while WB19 had an insertion in a gene encoding an SDR family NAD(P)-dependent oxidoreductase (unpublished data). The observed differences in antifungal activity may result from the random genomic integration of the *dmlR* gene, which could potentially affect different regulatory or metabolic pathways within the CP01 genome.

To delve deeper, we assessed the antifungal activity of the WT and the mutant WB12 by cultivating them in 50 mL and 4 L of King's B liquid media. The supernatant was boiled at 85°C for 10 minutes to eliminate any remaining viable cells. It was then tested for antifungal activity using an agar diffusion assay, as previously

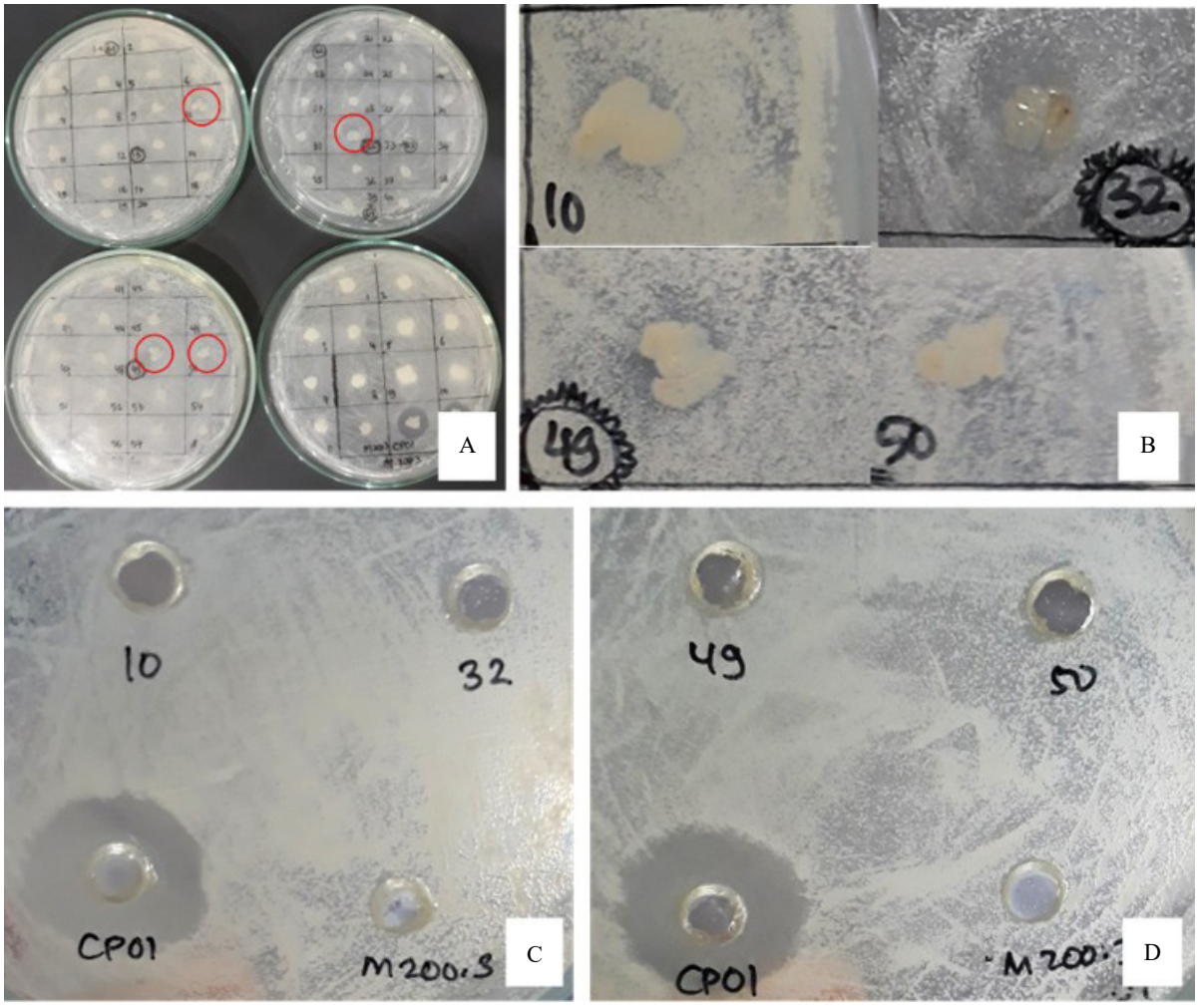

Figure 3. Antifungal activity assay of transconjugant from complementation study of DmlR on PDA spread with *S. cerevisiae*: (A) colony 10, 32, 49, and 50 (highlighted in red) presenting small clear zones; (B) magnification of colony 10, 32, 49, and 50; antifungal production of colony 10 and 32 (C), along with colony 49 and 50 (D) in King's B 50 mL, plated in PDA with yeast

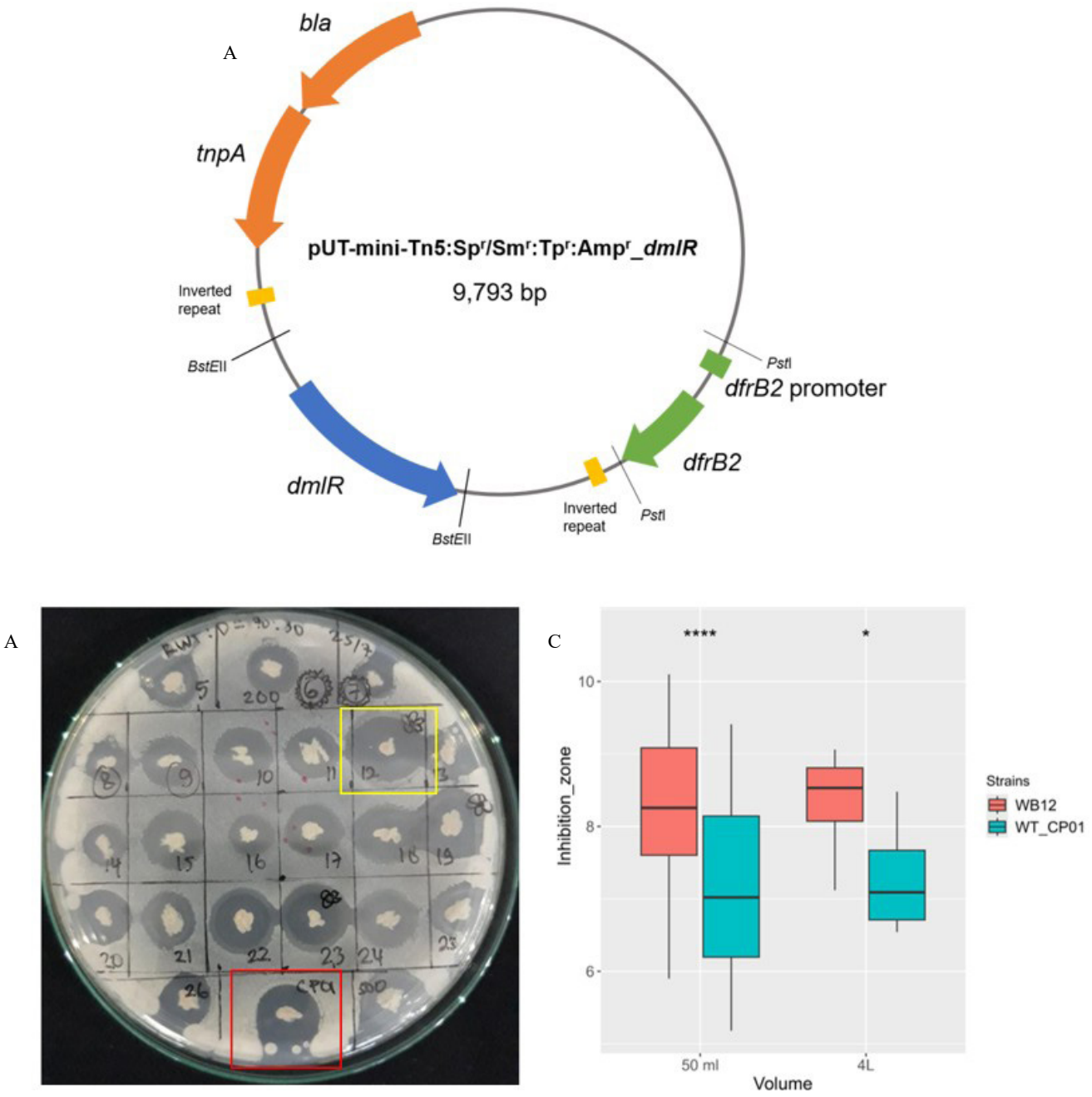

Figure 4. The *dmlR* gene was overexpressed in CP01 (A) plasmid construct, designed to generate DmlR overexpression strains. The construct incorporated the *dmlR* gene and the trimethoprim resistance gene (*dfrB2*) into the pUT-mini-Tn5 plasmid for random mutagenesis, (B) the antifungal activity of overexpressing mutant WB12 (highlighted in yellow box), was compared to *B. ubonensis* CP01 wild type (WT; highlighted in red box) against *Saccharomyces cerevisiae*, (C) antifungal production of WB12 and WT in 50 mL and 4 L in King's B medium. p-value: **** < 0.001, * < 0.05

described, and the resulting zone of inhibition was measured. The antifungal activity of the WB12 mutant consistently surpassed that of the wild-type CP01 strain as the scale of antifungal production increased from 50 mL to 4 L (Figure 4C).

On average, after 24 hours of antifungal production, the antifungal activity of WB12 mutant was found to be 15.46% higher compared to the CP01 wild-type strain. This difference was statistically significant, as shown in Table 6. No physiological changes or growth inhibition were observed in mutant WB12, as the growth rates (OD_{600}) of both strains during the exponential growth phase were nearly indistinguishable (Figure 5A). Additionally, the pH of the fermentation media remained consistent between the two strains (Figure 5B), further indicating that the overexpression of dmlR did not affect overall growth or fermentation conditions.

3.5. HPLC Analysis to Discern Distinct Antifungal Compounds

The antifungal production of both the WT and WB12 mutant strains was evaluated using High-Performance Liquid Chromatography (HPLC). Antifungal compounds were initially extracted from the cell-free culture through ammonium sulfate precipitation, following the optimized protocol identified an antifungal compound, referred to as component O, which eluted at 24–25 minutes in the HPLC profile of *Burkholderia ubonensis* CP01 wild type. This compound showed similarities to occidiofungin and burkholdine, as determined by liquid chromatography–electrospray ionization/mass spectrometry (LC-ESI/MS) analysis.

In this study, the compound elucidated at the same retention time was identified from the RP-HPLC chromatograms of both the CP01 wild type and the WB12 mutant. The WB12 mutant exhibited a similar peak with significantly higher intensity compared to CP01, as illustrated in Figure 6. Specifically, the area under the curve (AUC), expressed in milli-absorbance units multiplied by retention time (mAU*s), for WB12 at 25 minutes was 26220.4, representing a 1.11-fold increase over the wild-type AUC of 23593.1. The fraction containing this compound was tested for antifungal activity. Consistent with the AUC results, the CP01 wild type showed an antifungal inhibition zone of 7.97±0.97 mm, while the WB12 mutant demonstrated enhanced antifungal activity with an inhibition zone of 9.20±1.11 mm (Table 7). This indicates that the production of the antifungal compound in the WB12 mutant strain was approximately 15.43% higher than in the CP01 wild-type strain. The increase was statistically significant, as confirmed by the statistical analysis presented in Table 7.

Additionally, a new peak emerged in the elution window of 18-19 minutes for the WB12 mutant. While our primary focus was on enhancing the occidiofungin and burkholdine-like compound (eluting at 24-25 minutes), we did not further investigate this new peak. Additional studies are required to investigate its identity and potential antifungal activity. Some peaks were also absent in the WB12 strain, notably those at elution times of 20 and 23 minutes. This may indicate a downregulation in secondary metabolite synthesis. Their sequencing data identified 20 out of 23 biosynthetic gene clusters (BGCs), and of these, 13 were significantly affected by scmR, being either up- or down-regulated threefold or more. Ten were up- or down-regulated by fourfold or more. These findings highlight the crucial role of *dmlR* in regulating secondary metabolism in *B. ubonensis* CP01.

3.6. Localization of Mutated Genes in the Mutant WB12

Whole-genome sequencing revealed that the introduced *dmlR* gene was inserted into the locus on chromosome 2, spanning from 831,410 bp to 837,419 bp, in the WB12 mutant. In contrast, the native *dmlR* gene in the WT strain is situated on chromosome 1 (Figure 7). Analysis of gene insertion indicated that the introduced *dmlR* disrupted a gene belonging to a family of integral membrane permeases involved in gluconate uptake. Our findings provide strong support for the role of DmlR as a positive regulator in the biosynthetic pathway of antifungal production in Burkholderia ubonensis CP01. Furthermore, the inclusion of this

Table 6. Inhibition zone (mm) of *B. ubonensis* CP01 wild-type (WT) and mutant (WB12) against *Saccharomyces cerevisiae*

	WT	WB12	p-value
50 mL*	7.15±1.11	8.31±1.04	<0.001
4 L**	7.27±0.75	8.34±0.72	0.023

The result indicates the mean value ± standard deviation from three replicates. Data were subjected to ANOVA with Tukey's post hoc analysis. Significant if p-value<0.05

*The data were collected from three biological replicates and two technical replicates

**The data were collected from two biological replicates and two technical replicates

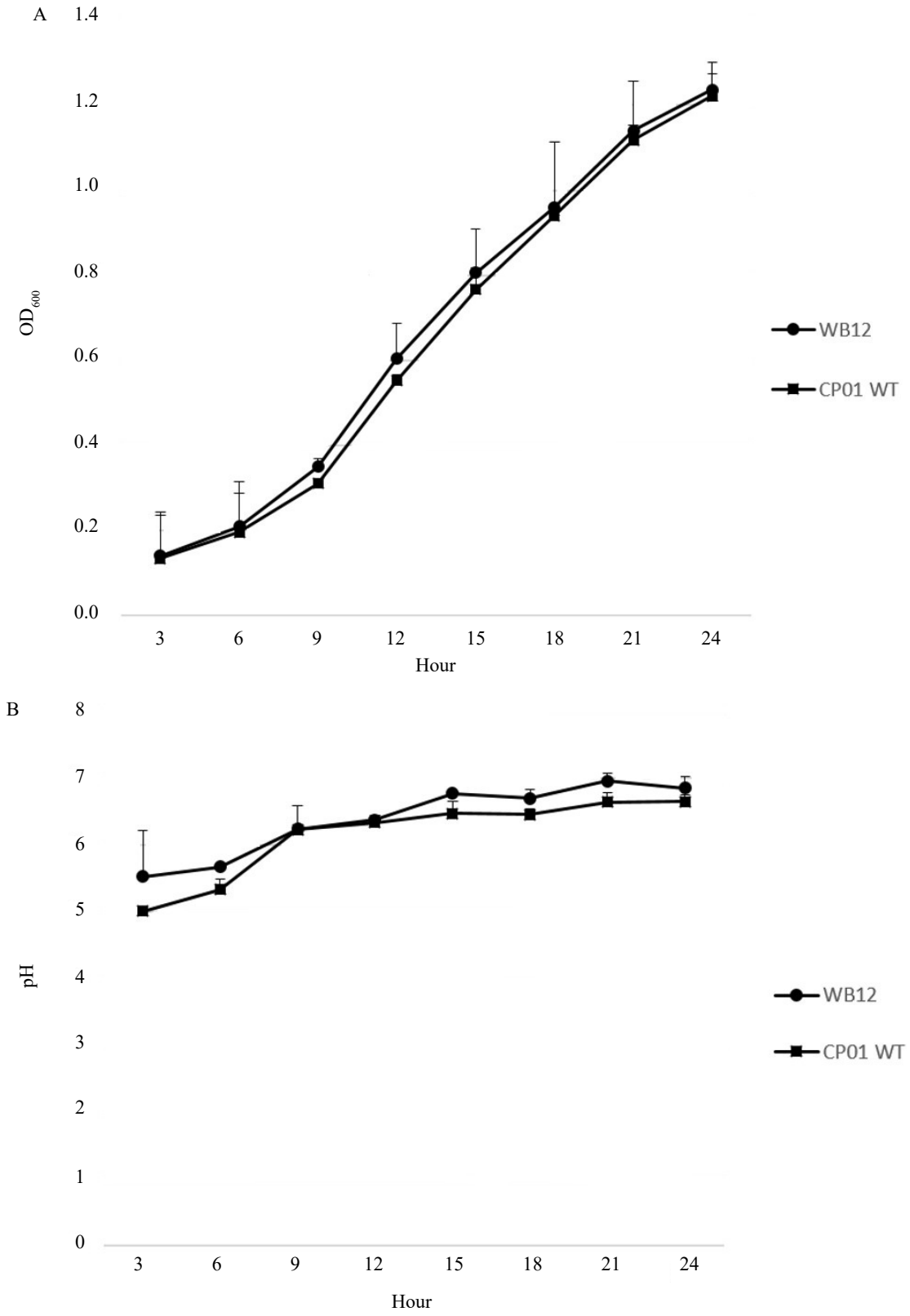

Figure 5. (A) OD_{600} and (B) pH, parameters of *B. ubonensis* CP01 wild-type (WT) and overexpressing dmlR gene mutant WB12 production over 24 hours in 4 L King's B medium. The data were obtained from two biological replications

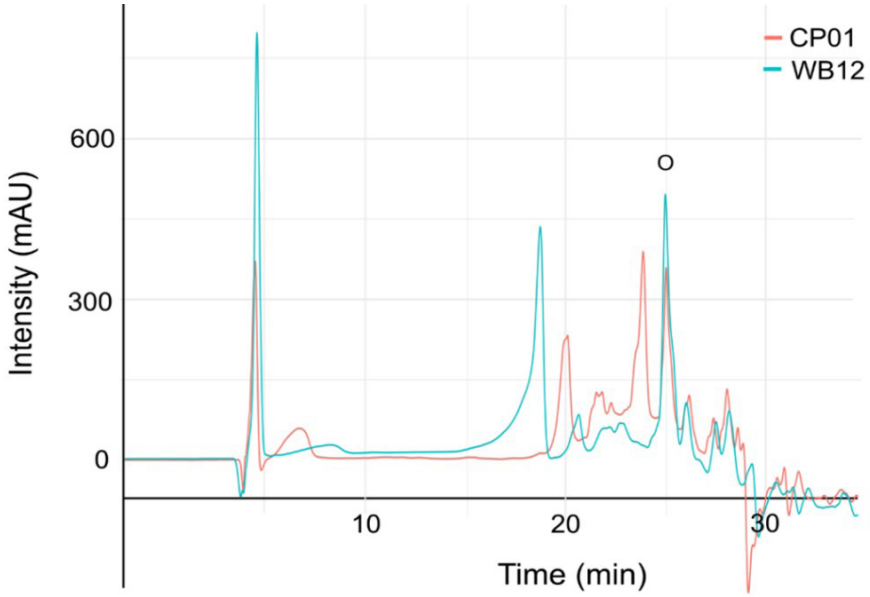

Figure 6. Comparison of distinct peaks corresponding to antifungal compounds between the *B. ubonenesis* CP01 WT (shown by red line) and WB12 strains (shown by blue line) using RP-HPLC analysis

Table 7. Inhibition zone (mm) of the fraction-of-interest (peak O) from *B. ubonensis* CP01 wild-type (WT) and mutant (WB12) strains

Strain	Mean of inhibition zone (mm)	p-value
CP01	7.97±0.97	0.014
WB12	9.20±1.11	

The result indicates the mean value ± standard deviation from 2 biological replications. Data were subjected to the Mann-Whitney U test. Significant if p-value < 0.05

gene presents a promising strategy for enhancing the production of this compound.

4. Discussion

The genera *Burkholderia* are found in various environments, including soil, plants, and some species inhabit the bodies of animals and humans, either as pathogens or as symbionts. Some *Burkholderia* species are reported to have potential as biocontrol agents due to their ability to produce various secondary metabolites, including compounds with antibacterial, antifungal, insecticidal, and herbicidal properties. A key component of *Burkholderia's* biocontrol properties is the presence of biosynthetic gene clusters (BGCs). However, most of these BGCs are "silent" or "cryptic" and do not give rise to detectable levels of secondary metabolites under normal laboratory conditions. Genetic manipulation of the LysR-type transcriptional regulator ScmR in *B. thailandensis* has been shown to successfully alter the metabolic profile, including the activation of the thailandamide biosynthesis gene cluster (Ishida *et al.* 2010). Moreover, homologs of ScmR are also found to regulate secondary biosynthetic metabolism, including the production of malleilactone by *B. pseudomallei* (Thapa and Grove 2019). LTTRs are among the most prevalent transcriptional regulators in prokaryotes, capable of functioning as either positive or negative modulators for a wide array of genes associated with secondary metabolism (Wu *et al.* 2023). The improvement effect generated from this regulatory sequence manipulation has also been notable, implying its promising potential as a prevalent key regulator for secondary metabolite production in the *Burkholderia* genera.

Previously, we have found that the secondary metabolite produced by *Burkholderia ubonensis* CP01 possesses antifungal activity and holds significant promise for effectively managing BSR disease in oil palm plantations (Prihatna *et al.* 2022). In our pursuit of enhancing strains that produce this antifungal agent, we have searched and genetically manipulated a ScmR regulatory element homolog found in CP01, namely DmlR. Knockout of the *dmlR* gene in this bacterium led to a complete absence of antifungal production. The complementation study failed to restore the antifungal activity of *B. ubonensis* CP01, likely due to ineffective antibiotic selection caused by the bacterium's intrinsic resistance to multiple antibiotics. Our findings were consistent with those reported by Rhodes and Schweizer (2016), who noted that many *Burkholderia*

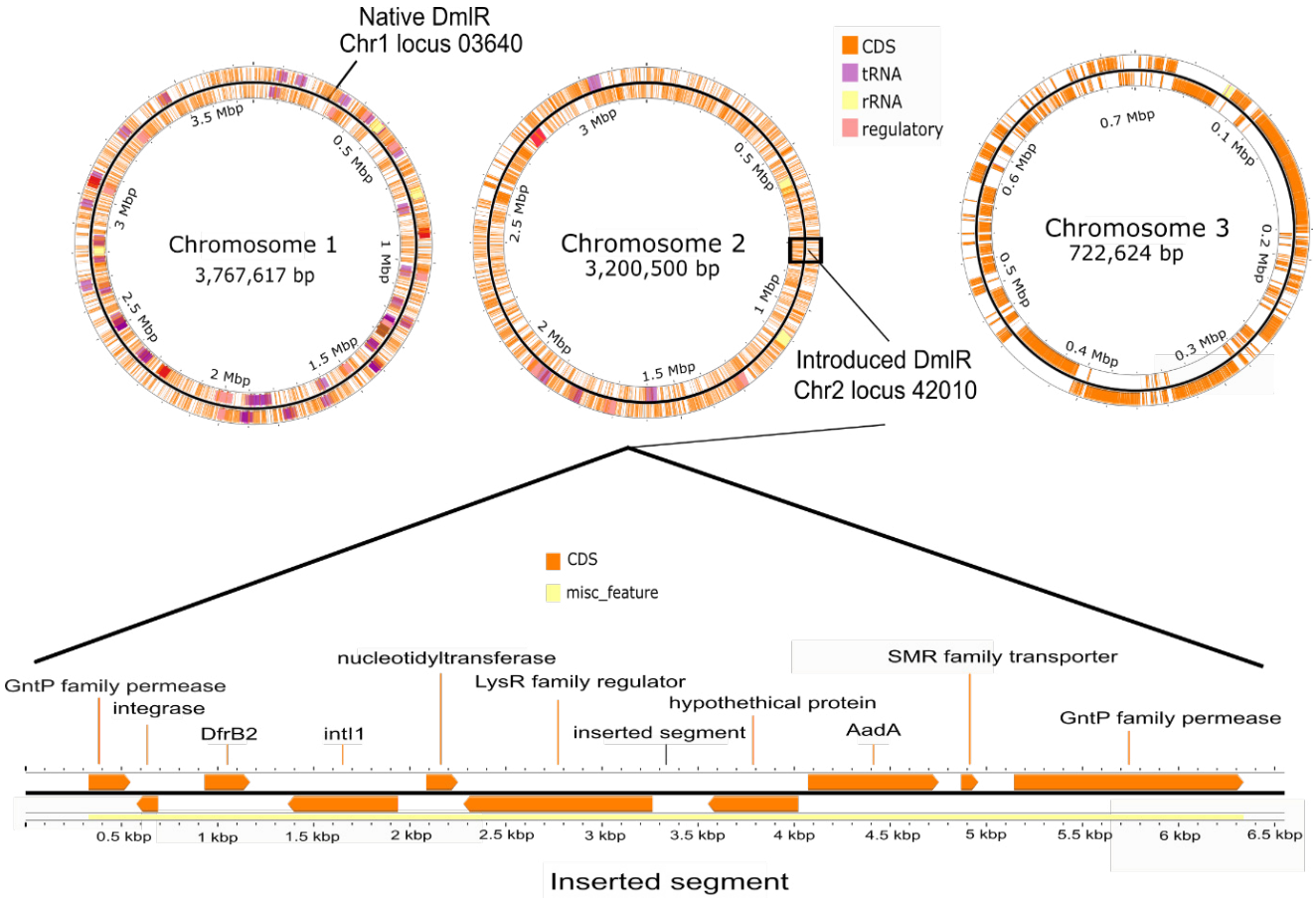

Figure 7. Whole-genome sequencing reveals the location of the inserted *dmlR* gene on chromosome 2 of *B. ubonensis* CP01

species have acquired resistance to numerous antibiotics, often due to mechanisms like reduced outer membrane permeability and efflux pumps. Similarly, a previous study discovered the tetR(64)-tetA(64) genes located in a region of chromosome 1, which is responsible for high tetracycline-concentration resistance in *B. ubonensis* (Somprasong *et al.* 2021). The authors postulated that these genes may have been acquired through homologous recombination after the horizontal gene transfer protocol. Despite the use of high antibiotic concentrations, a stable complemented mutant could not be obtained, suggesting that the plasmid was lost during subculture. As a result, we decided to pursue overexpression of the *dmlR* gene. Upon overexpression, there was a significant increase in antifungal production, indicating that DmlR acts as a positive regulator of the antifungal genes in *B. ubonensis* CP01.

By overexpressing the *dmlR* gene, a potential mutant strain (WB12) was developed to enhance the production of an antifungal compound. This variant demonstrated the ability to produce 15.46% more antifungal activity than the unmodified strain, as determined by the agar well diffusion assay after 24 hours of cultivation in both 50 mL and 4 L media. Moreover, no physiological changes or growth inhibition were observed in mutant WB12. This mutagenic strain demonstrates enhanced antifungal activity, suggesting its potential for future applications in improving production efficiency. However, further studies are required to assess the strain's production scalability and perform cost-benefit analyses to validate its potential for industrial application. Previous studies have demonstrated that augmenting the expression of LTTRs can lead to enhancements in the production of antimicrobial secondary metabolites (Barajas *et al.* 2017; Zhou *et al.* 2020). For instance, the overexpression of the LysR family transcriptional regulator fkbR1 led to a 33.5% increase in ascomycin production in *Streptomyces hygroscopicus* (Song *et al.* 2017).

Consistent with previous findings, HPLC analysis revealed that the production of antifungal compound

in the mutant strain WB12, eluted at a retention time of 25 minutes and confirmed by agar diffusion assay, was 15.43% significantly higher compared to the wild-type strain. Additionally, the area under the curve (AUC) at this retention time in WB12 was 1.11-fold greater compared to the CP01 wild-type strain.

Intriguingly, a peak observed in *B. ubonensis* CP01 wild-type prior to the occidiofungin and burkholdine-associated (O) peak was absent in WB12, indicating that the mutant strain did not exhibit the double-peak pattern seen in the wild-type chromatogram. Although the nature of this pattern has not been fully characterized, similar observations were reported by Ravichandran *et al.* (2013). During RP-HPLC purification of occidiofungin, three peaks were observed, with occidiofungin eluting as a double peak preceding a third peak. Notably, one of the early peaks does not exhibit antifungal activity. This author suggested that the mutation might be responsible for structural variations that lead to an increased antifungal peak. In our study, the absence of the earlier peak in WB12 may reflect changes in the biosynthetic pathway or metabolite profile, but further structural or compositional analysis, such as mass spectrometry, UV spectroscopy, or nuclear magnetic resonance (NMR), is needed to clarify the nature of this difference. Therefore, although the altered peak pattern coincides with enhanced antifungal activity, its underlying cause remains unclear and requires further chemical characterization before any structural implications can be drawn.

Similar to other LTTRs, it is likely that DmlR regulates numerous genes through various, potentially overlapping mechanisms, where one mechanism might influence or interact with other mechanisms. For example, a previous study observed that FinR, the LysR-family regulator, exhibited the ability to control the expression of 11 distinct gene clusters (Xiao *et al.* 2018). Consequently, it becomes crucial to delve deeper into the molecular mechanisms behind DmlR's actions to characterize genes directly controlled by DmlR and those influenced indirectly. Identifying the most relevant genes and pathways, combined with optimization of culture conditions, has the potential to yield enhanced production of the antifungal compound from *B. ubonensis* CP01 for agricultural applications.

In conclusion, this study identified and genetically manipulated *dmlR*, a putative *scmR* homolog in *B. ubonensis* CP01. This manipulation led to the creation of both knockout and overproducing strains. The knockout strains exhibited no antifungal activity, while the introduction of this LTTR resulted in the overproduction of an antifungal compound. While limited to a single gene and phenotype, this finding provides preliminary insight into the potential regulatory role of the dmlR gene, an LTTR, in modulating antifungal activity in *Burkholderia* species. To enhance antifungal activity and potentially activate dormant BGCs, further exploration of the molecular mechanisms governing DmlR's actions is essential. This includes characterizing genes directly controlled by DmlR and those indirectly influenced by it. In summary, despite the absence of in planta and field validation, this engineered strain may represent a promising early-stage candidate for developing microbial biocontrol agents against oil palm diseases.

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