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RESEARCH

Identification of strong constitutive promoters in *Burkholderia stagnalis* TBRC 18363 for activating natural product production in Gram-negative bacteria

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Abstract

Gram-negative bacteria are emerging as an important source of natural products with pharmaceutical potential. However, the limited availability of genetic tools for drug discovery and sustainable production of secondary metabolites remains a challenge. *Burkholderia* spp. serve as a promising source for such tools, as these bacteria produce diverse natural products and are amenable to genetic modification. We sequenced the genome of *Burkholderia stagnalis* TBRC 18363 and performed transcriptomic analysis to identify genes highly expressed in early to late exponential cultures. We hypothesized that the sequences upstream of the most highly expressed genes contain strong and constitutive promoters active in heterologous Gram-negative hosts. Twenty-six *B. stagnalis* TBRC 18363 promoters were evaluated in *Escherichia coli* and *Pseudomonas putida* reporter systems. Two promoters, p2035 and p5642, exhibited superior performance in both systems. Promoter exchange experiments at biosynthetic gene clusters showed that these promoters can enhance the production titers of icosalide in *B. stagnalis* TBRC 18363 and FR900359, a $G_{q/11}$ protein inhibitor depsipeptide, in *Chromobacterium vaccinii*. Therefore, the p2035 and p5642 promoters are applicable for target gene overexpression in Gram-negative bacteria and can serve as tools for unlocking the potential of cryptic biosynthetic genes.

Key points

- *Strong constitutive promoters of Burkholderia stagnalis TBRC 18363 were identified.*
- *Efficiencies of the selected promoters were evaluated in two heterologous hosts.*
- *p2035 and p5642 promoters boosted BGC expression in Burkholderia and Chromobacterium.*

Keywords *Burkholderia* · Promoter · RNAseq · Icosalide · $G_{q/11}$ protein inhibitor · *Chromobacterium*

Introduction

Bacterial natural products (NPs) represent a valuable source of bioactive compounds pivotal for the advancement of pharmaceutical and agricultural technologies. These secondary metabolites have led to the development of life-saving antibiotics, antifungals, anticancer agents, and various agrochemicals. Actinomycetes are the most prominent bacterial source of biologically active NPs, which are responsible for approximately 70% of the antibiotics used as human therapeutics (Bérdy 2005; Behie et al. 2017). This dominance is attributed to the high diversity of biosynthetic gene clusters (BGCs) present in actinomycetes, many of which have been successfully harnessed

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for drug discovery (Belknap et al. 2020). These bacteria have been extensively studied for decades, and comprehensive genetic manipulation tools have been established to activate and engineer their NP biosynthetic pathways (Kieser 2000).

In the last 20 years, additional taxonomic groups have emerged as excellent NP producers from which bioactive compounds can be isolated (Masschelein et al. 2017; Wilson and Brimble 2021). Furthermore, genome mining suggests that bacterial NP producers are far more prevalent than previously thought (Herrmann et al. 2017). Specifically, the exploration of previously underexplored Gram-negative bacteria has revealed a reservoir of cryptic biosynthetic gene clusters (BGCs), many of which remain silent under standard laboratory conditions. This cryptic nature has generated significant interest, as these gene clusters harbor the potential to produce novel bioactive compounds. Among Gram-negative bacteria, species in the class Betaproteobacteria, particularly from the genera *Burkholderia* and *Chromobacterium*, have attracted considerable attention due to the isolation of novel bioactive natural products like chromodepsins and to the abundance of cryptic BGCs for which no product has been detected (Kunakom and Eustáquio 2019). While the biosynthetic capacity of these Gram-negative bacteria rivals that of actinomycetes and bears the potential for novel chemistry, the development of effective genetic tools for the activation and manipulation of NP biosynthetic pathways in these organisms has lagged behind. Unlike actinomycetes, where well-characterized promoters, regulatory elements, and vectors exist for manipulating NP biosynthesis, tools for Gram-negative bacteria, particularly Betaproteobacteria, are limited. A key component of any genetic toolbox is a validated set of strong constitutive promoters that can reliably drive gene expression in the relevant taxonomic group. In the context of NP production, activation of a previously silent gene cluster could lead to the discovery of a novel NP, or an increase in the respective secondary metabolite for improving the production process. The constitutive promoter upstream of the S7 ribosomal protein gene from *Burkholderia* sp. LB400 was used to drive reporter gene expression in *Burkholderia cepacia* (Lefebre and Valvano 2002), and constitutive promoters from *Burkholderia pyrrocinia* JK-SH007 were shown to drive overexpression of fluorescent protein reporters in *Burkholderia multivorans* WS-FJ9 and *Escherichia coli* S17-1 (Wu et al. 2023). None of these *Burkholderia* promoters have been tested for driving the expression of an NP biosynthetic gene cluster. Constitutively expressed promoters from *Schlegelella brevitalea* DSM 7029, a strain in the Burkholderiales order, have been identified and shown to drive overexpression of BGCs in the same strain for enhancing the production of epothilones and rhizomide (Ouyang et al. 2020). Another example is Php, a strong promoter of a hypothetical protein, in *Burkholderia* sp. JP2-270 that was evaluated via the enhanced green fluorescent protein (EGFP) reporter and could overexpress endogenous pyrrolnitrin biosynthetic genes (Ke et al. 2024). Nevertheless, these promoters have not yet been tested for activating BGC expression and NP production in other Gram-negative bacteria, leaving a gap in the genetic toolkit available for NP research in this group.

The need for a broader range of strong constitutive promoters is underscored by the increasing complexity of synthetic biology approaches for NP production. For instance, research involving promoter refactoring across multiple genes within a BGC requires a variety of promoters to fine-tune expression levels of each gene. In Betaproteobacteria, where many cryptic BGCs somehow remain silent, the ability to test a wide array of strong promoters is especially important to achieve robust expression of the desired pathways. In such cases, the ability to test a wide array of strong promoters ensures more precise control over gene expression, which is critical for maximizing NP yield. Moreover, the selection from a promoter panel directly influences the biosynthetic output of engineered strains, as a promoter that works well for one gene may not be optimal for another due to differences in context-dependent gene regulation. This increased flexibility enables researchers to test various promoter-gene combinations, which could lead to significantly improved production of valuable secondary metabolites. In this study, we aimed to identify strong and constitutive promoters in an NP-producing *Burkholderia* strain with abundant BGCs. We tested these promoters for their abilities to activate the production of secondary metabolites, thereby expanding the genetic toolbox for NP biosynthesis in Gram-negative bacteria. In addition to evaluating the promoters in other *Burkholderia* strains, we tested the most promising *Burkholderia* promoter in a different genus and were able to enhance the production of the pharmaceutical relevant chromodepsins.

Materials and methods

Bacterial strains, media, and chemicals

Burkholderia stagnalis TBRC 18363 was obtained from the Thailand Bioresource Research Center (TBRC). *Escherichia coli* DH5α (Thermo Fisher Scientific, USA) was used for all plasmid constructions. *E. coli* BL21(DE3) (Thermo Fisher Scientific, USA) and *Pseudomonas putida* KT2440 (ATCC® 47,054™) were used as host strains for promoter characterization. The bacterial strain *Chromobacterium vaccinii* DSM 25150 (= ATCC BAA-2314, = NRRL B50840, = MWU205) was obtained from the German collection of microorganisms and cell cultures DSMZ. For conjugation experiments, *E. coli*

ST18 (Thoma and Schobert 2009) was used as a donor of plasmids. *E. coli* DH5α, BL21(DE3), and ST18, and *P. putida* KT2440 were cultivated in Luria–Bertani (LB) medium (Sambrook and Russell 2001) supplemented with antibiotics (50 µg/mL ampicillin (Sigma, USA), 50 µg/mL kanamycin (Sigma, USA), and 15 µg/mL tetracycline (Fluka, China) as appropriate). *B. stagnalis* TBRC 18363 was first revived from glycerol stock on LB medium, followed by cultivation in LS2 (Boonlarppradab et al. 2011) for RNA-seq analysis and BMPM (Zhang et al. 2016) for icosalide production. *C. vaccinii* was grown in LB-MOPS medium (peptone 10 g/L, yeast extract 5 g/L, sodium chloride 5 g/L, MOPS 10.5 g/L, pH 7.0). A total of 100 µg/mL apramycin (Sigma, USA) was added where necessary.

Genomic DNA extraction and genome sequencing

Genomic DNA of *B. stagnalis* TBRC 18363 was extracted using the protocol described by Green et al. (2012) and subsequently purified using the Quick-DNA Fungal/Bacterial Miniprep kit (Zymo Research, USA). Genomic DNA quality was assessed by agarose gel electrophoresis and Nanodrop spectrophotometer (Thermo Scientific, USA). Genome sequencing was performed using a MinION system (Oxford Nanopore Technologies Ltd., Oxford, UK) and Illumina HiSeq X sequencing (150 bp) by NovogeneAIT Genomics, Singapore. Genome assembly was performed using Unicycler (Wick et al. 2017). QUAST version 4.1 (Gurevich et al. 2013) was applied for the evaluation of the assembled genome sequence quality. Genome completeness was measured using BUSCO version 5.4.2 (Manni et al. 2021). The assembled genome was submitted to NCBI under BioProject number PRJNA1068327. Biosynthetic gene clusters were annotated by antiSMASH 7.0 (Blin et al. 2023).

RNA preparation and RNAseq analysis

RNA was extracted from *B. stagnalis* TBRC 18363 using the RiboPure™ RNA Purification Kit, bacteria (Invitrogen, USA), followed by DNase I treatment. RNA concentration and quality were assessed by Nanodrop and agarose gel electrophoresis. RNA samples were subsequently submitted for RNA-seq (NovogeneAIT Genomics, Singapore). The integrity of RNA samples was assessed by Agilent 2100 bioanalyzer. RNAseq was performed on an Illumina NovaSeq 6000 with 150 bp paired end reads yielding 3 GB data or more per sample. The RNAseq raw data were processed as follows. Adapter sequences were trimmed by FASTP (Chen et al. 2018). Sequence quality was assessed and reported by FASTQC (Andrews 2010) and MultiQC (Ewels et al. 2016), respectively. The processed RNA sequences were mapped to the genome sequences of *B. stagnalis* TBRC 18363 by HISAT2 (Kim et al. 2015), Sequence Alignment Map (SAM) format, then subsequently converted and sorted to Binary Alignment Map (BAM) format using samtools (Danecek et al. 2021). The BAM files were subsequently processed using Picard (http://broadinstitute.github.io/picard/; Broad Institute, accessed 18 May 2021) to mark and remove sequence duplicates. The table of processed mapped read counts to each feature was obtained using the htseq-count command in the HTseq (Anders et al. 2015) package. The read count table was provided as input to DESeq2 (Love et al. 2014), which was used to generate a Principal Component Analysis (PCA) plot of the samples. Read counts of each gene were normalized to Transcripts Per Million (TPM) by StringTie (Kovaka et al. 2019), using the -G option with gene annotations generated by RAST (Aziz et al. 2008). The ranked TPM data were used to identify promoter candidates. The RNAseq data were submitted to the NCBI Sequence Read Archive with Gene Expression Omnibus (GEO) accession number GSE269653.

Transformation of reporter plasmids into *E. coli* BL21(DE3) and *P. putida* KT2440

Reporter plasmid constructions are described in Supplementary methods. All reporter plasmids were transformed into *E. coli* BL21(DE3) or *P. putida* KT2440 by electroporation. The transformants were selected on LB agar supplemented with 50 µg/mL ampicillin or 15 µg/mL tetracycline, respectively.

Detection of fluorescence in *E. coli* BL21(DE3) and *P. putida* KT2440 harboring EGFP reporter plasmids

Five microliters of *E. coli* BL21(DE3) and *P. putida* KT2440 transformants from glycerol stock were added to 3 mL LB supplemented with antibiotics and cultivated overnight at 37 °C and 30 °C, respectively. The overnight cultures were diluted in fresh LB media supplemented with antibiotics to an OD_{600} of 0.01 and two hundred microliters were aliquoted into three wells of a 96-well plate. No IPTG was added in any reporter experiment. The plates were then incubated at 37 °C or 30 °C and 800 rpm for 8 h. OD_{600} and EGFP fluorescence of whole cell cultures were measured using a Microplate Reader (BioTek Synergy H1, USA). Fluorescence signal was detected at the excitation and emission wavelengths of 485 and 528 nm, respectively. Average fluorescence intensity and OD_{600} values from at least three independent repeats were used for data analysis. The fluorescence intensity of each promoter transformant was normalized with its corresponding OD_{600} value. The relative normalized intensity was calculated by dividing the normalized fluorescence intensity of the test promoter

transformant by the normalized fluorescence intensity of the transformant with pET17b or pTet-T7 control plasmid. Relative normalized intensity values greater than 1 indicated promoter activity, whereas values equal to or less than 1 indicated the absence of promoter activity.

Promoter exchange in icosalide and FR900359 BGCs

Construction of conjugative plasmids for promoter exchange in icosalide and FR900359 BGCs is described in the Supplementary methods. All conjugation constructs were transformed into *E. coli* ST18 by electroporation. Transformants were selected on LB agar containing 50 µg/mL 5-aminolevulinic acid (5ALA) (Zymo Research, USA) and antibiotics according to the corresponding constructs (15 µg/mL tetracycline and 25 µg/mL chloramphenicol (Sigma, Germany) for *B. stagnalis* and 100 µg/mL apramycin for *C. vaccinii*). For conjugation of *B. stagnalis* TBRC 18363, 500 µL overnight culture of *B. stagnalis* (LB medium, 30 °C) and *E. coli* ST18 harboring the conjugation plasmid (LB medium supplemented with 50 µg/mL 5ALA, 15 µg/mL tetracycline, and 25 µg/mL chloramphenicol, 37 °C) were inoculated into 25 mL of LB medium and LB containing 50 µg/mL 5ALA, 15 µg/mL tetracycline, and 25 µg/mL chloramphenicol, respectively. The cultures of *B. stagnalis* TBRC 18363 and *E. coli* ST18 were cultivated at 30 °C and 37 °C, respectively, until an OD_{600} of 0.6–0.8 was reached. Both cultures were harvested by centrifugation, washed with 10 mL fresh LB medium twice, and resuspended in 1 mL LB medium. *E. coli* and *B. stagnalis* were mixed in a ratio of 1:3 and spotted on an LB agar plate containing 50 µg/mL 5ALA. After an initial incubation at 37 °C for 2 h, the plate was transferred to 30 °C and incubated overnight. On the next day, the spot was resuspended in 1 mL LB medium, and 10^{-2} and 10^{-3} dilutions were plated onto LB agar containing 200 µg/mL chloramphenicol. Plates were incubated at 30 °C until single colonies appeared (1–2 days). Single exconjugants were streaked onto LB agar plates supplemented with 200 µg/mL chloramphenicol and incubated overnight at 30 °C. Conjugation of *C. vaccinii* was done in a similar manner with the exception that the incubation temperature was shifted to 28 °C and the selection for mutants was done on LB agar plates containing 100 µg/mL apramycin. In both hosts, promoter exchange occurred via single-crossover homologous recombination between the chromosomal 5′ region of the target biosynthetic gene and the matching homology fragment present on the conjugative plasmid, following the integration strategy described in Pistorius et al. 2021. Recombinant strains were identified by colony PCR using verification primers listed in Table S5.

LC–MS/MS analysis of icosalide production in *B. stagnalis* TRBC 18363 and FR900359 production in *C. vaccinii*

Cells of *B. stagnalis* TRBC 18363 wild type and *ics*-promoter activated mutants were streaked out on LB agar plates with or without 200 µg/ml chloramphenicol and incubated overnight at 30 °C. One loop of cells from each plate was used to inoculate 1 mL LB medium with or without chloramphenicol and cultivated overnight at 30 °C at 200 rpm. On the next day, 1% of the overnight culture was used to inoculate 10 mL BMPM medium without antibiotic and incubated at 30 °C with 200 rpm shaking for 3 days. The cultures were extracted with 10 mL ethyl acetate mixed with acetone in the ratio 15:2 v/v with shaking for 2 h. After centrifugation, 1 mL sample of the extract layer was transferred to a clean tube and evaporated at RT. Dried extracts were resuspended in 150 µL methanol, filtered, and analyzed by LC–MS/MS. The LC gradients, MS acquisition, data processing workflow, and compound annotations are described in the Supplementary methods.

For cultivation of *C. vaccinii* and p2035 promoter mutants, 1 mL of cryo-preserved cell suspension was used to inoculate 25 mL LB-MOPS in a shake flask with or without 100 µg/mL apramycin. The cells were initially cultivated for 12–16 h at 28 °C on an orbital shaker with 50 mm amplitude at 200 rpm. The main culture was run in 24-deep well plates with 2 mL LB-MOPS medium without antibiotic inoculated with 1% of the preculture and incubated for 72 h at 28 °C on an orbital shaker with 50-mm amplitude at 250 rpm. All cultures were performed in triplicate. Upon harvest, the culture broths were frozen and kept at − 20 °C for at least 24 h. Four milliliter of a mixture of acetonitrile/water (3:1), 0.75% formic acid, were added to each well of freeze-thawed culture broth and shaken for 10 min at 1000 rpm. A total of 4.5 mL thereof were transferred to a fresh 24-deepwell plate containing 1.12 g of magnesium sulfate and 0.28 g of sodium acetate per well and shaken vigorously for 10 min at 1000 rpm. The mixture was centrifuged at 1000 rpm for 15 min to separate the phases. A 1.2 mL sample of the organic layer was transferred to a 96-deepwell plate and the solvent was removed under vacuum. The resulting residue was dissolved in DMSO and subjected to UHPLC–MS analysis. Quantification of FR900359 was performed by UV detection at 220 nm using external standards. The FR900359 standard used in this study was isolated from culture broth of *C. vaccinii* following the procedure described by Pistorius et al. (2021). Detailed cultivation conditions, extraction

procedures, and UHPLC–MS parameters are provided in the Supplementary Methods.

Results

Identification of strong constitutive promoters of *B. stagnalis* TBRC 18363

We selected an uncharacterized *Burkholderia* strain from our collection (TBRC 18363) as a strain representative of this genus due to its broad-spectrum antifungal activity against economically important plant pathogens including *Alternaria brassicicola* causing leaf spot in brassica crops, *Curvularia lunata* causing rice dirty panicle and *Colletotrichum acutatum* causing chili anthracnose disease (Table S1). The strain was subjected to genome sequencing using Illumina and Nanopore platforms. The hybrid assembled genome of 8,006,692 bp comprised four contigs with an overall GC content of 67.3%, including a total of 7,621 coding sequences and 87 RNA genes annotated by RAST (Aziz et al. 2008) (Table S2). Average nucleotide identity by BLAST (ANIm) analysis showed that the selected strain has the highest identities to *Burkholderia stagnalis* CCUG 65686 ^{T} (GCF_008802125, 94.17% identity). The selected strain is consequently named as *B. stagnalis* TBRC 18363. The genome was split among three chromosomes, as is commonly found in *Burkholderia* species (Esmaeel et al. 2016). Genome analysis using antiSMASH 7.0 (Blin et al. 2023) indicated 20 putative BGCs in the genome (Table S2). Among these BGCs, high similarity to BGCs for the synthesis of known compounds included occidiofungin (94% similarity), ornibactin (100% similarity), and icosalide (100% similarity).

The *B. stagnalis* TBRC 18363 transcriptome was investigated by RNAseq. *B. stagnalis* TBRC 18363 was cultured in LS2 medium at 25 and 30 °C, and RNA was extracted from the harvested cells after 24, 30, and 48 h. The sampled time points corresponded to early-, mid- and late-exponential phase, respectively (Fig. S1). RNA samples collected from three independent cultures were submitted for RNAseq service (Novogene). Principal component analysis (PCA) indicated grouping of replicate samples by culture condition (Fig. S2). RNAseq reads were mapped to 7621 annotated genes; 36 genes with no read count data were filtered out. The remaining 7585 genes were further analyzed to identify strong and/or constitutive promoters. Genes were ranked by expression level, and the top-ranked genes were selected as candidate genes for promoter characterization. Twenty-one genes displaying constitutively high expression across the sampled conditions were prioritized, which included ten genes annotated as encoding hypothetical proteins. Besides these highly-expressed genes, another five genes representing low-expressed genes were selected as controls. Notably, these low-expressed genes were part of annotated BGCs. All selected genes for promoter characterization, along with their full expression profiles across all six RNA-seq conditions, are provided in Table S3.

The intergenic region sequences upstream of the selected genes to the next adjacent gene were extracted for identifying putative promoters. Where the intergenic region exceeded 500 bp, the length of the promoter was adjusted to that size counting from the start codon of the downstream located gene. The intergenic regions were analyzed using Promotech (Chevez-Guardado and Peña-Castillo 2021). This promoter recognition tool uses a random forest model and trained data with hot-encoded features (RF-HOT). It was reported as a suitable tool for detecting bacterial promoters in a species-agnostic manner and outperforms other prediction tools (Chevez-Guardado and Peña-Castillo 2021). To predict promoter sequences using the 40 bp prediction option, all possible 40 bp sequences along the full length of each promoter were generated. Top-scoring Promotech promoters are listed in Table S4. It is noteworthy that not all promoter sequences identified by Promotech contained the typical − 10 and − 35 promoter motifs. In addition, Promotech could not detect promoter sequences upstream of genes 705, 932, 1957, 2082, 5120, 5617, 7122, 7217, 7456, and 7459.

Evaluation of *B. stagnalis* TBRC 18363 promoters in *E. coli* BL21 (DE3) and *P. putida* KT2440 host systems

To assess the functionality of the *B. stagnalis* TBRC 18363 promoters in heterologous hosts, the activities of the promoters for driving EGFP reporter gene expressions in *E. coli* BL21(DE3) were assessed. Sequences of reporter genes and primers for plasmid constructions are listed in Table S4 and S5. *E. coli* BL21 (DE3) EGFP transformants carrying p5642, p2035, p5617, p2571, and p1173 promoters upstream of *egfp* demonstrated the highest relative EGFP intensities compared with the other *Burkholderia* promoters. However, none of the *Burkholderia* promoters was stronger than the inducible T7 positive control promoter (Fig. 1a and Table S6).

The activities of *B. stagnalis* TBRC 18363 promoters were also evaluated in *P. putida* KT2440, another well-known Gram-negative bacterial host for heterologous gene expression. All materials for plasmid constructions of the *P. putida* KT2440 system are listed in Table S4 and S5. Among the tested *B. stagnalis* TBRC 18363 promoters, p2035, p5642, and p2111, as well as the T7 reference

promoter, showed high activities in the *P. putida* KT2440-EGFP system. In contrast, p705, p3303, p5330, 7459, p1173, p2571, and p5617 showed markedly lower activities and were assigned as weak to moderate promoters (Fig. 1b and Table S6). In both *E. coli* and *P. putida* heterologous host systems, the p2035 and p5642 promoters—located upstream of highly expressed genes annotated as hypothetical proteins (Table S3)—exhibited superior activities compared with the other tested *Burkholderia* promoters, suggesting that they are promising candidates for BGC activation/enhancement in a wide range of Gram-negative hosts.

Activation of biosynthetic gene cluster of icosalide in *B. stagnalis* TBRC 11456 and FR900359 in *Chromobacterium vaccinii* DSM 25150

The p2035 and p5642 promoters were tested for their potential to activate/boost NP production in two different systems. First, the capability of the promoters to activate a BGC in the original host *B. stagnalis* TBRC 18363 was evaluated. *B. stagnalis* TBRC 18363 harbors a BGC for icosalide, which corresponds to a single non-ribosomal peptide synthetase gene *ics* (15.10 kb). The native *ics* promoter was replaced with p2035 or p5642 via homologous recombination, and the extracts of the corresponding mutants were analyzed for icosalide production using high-resolution MS/MS fragmentation. The production of icosalide A1 was confirmed by LC–MS/MS analysis (Fig. S5 and Supplementary method). Both p2035 and p5642 promoter knock-in mutants produced icosalide A1 (Fig. 2a) at levels approximately 1.6- and 1.4-folds greater than the "no promoter" recombination control, respectively (Fig. 2b). This control strain was generated using the same single-crossover integrating procedure.

In the second approach, p2035 was tested for its capability to boost production of the pharmaceutically relevant compound FR900359 (Fig. 2c) in the Gram-negative strain *Chromobacterium vaccinii* DSM 25150 (Hermes et al. 2021). This cyclic depsipeptide is a potent and highly selective inhibitor of $G_{q/11}$ mediated signal transduction of associated G protein-coupled receptors (GPCRs) and has been explored as a lead molecule for targeted cancer therapy for uveal melanoma and other cancers which harbor GNAQ or GNA11 (encoding G_q or G_{11}) oncogenic mutations (Onken et al. 2018). The corresponding BGC spans approximately 35 kb and is organized as one operon consisting of five non-ribosomal peptide synthetase genes and three additional modifying enzymes. The native FR promoter was replaced with p2035 via homologous recombination, and two mutant clones were compared with the wild type for FR900359 production (Pistorius et al. 2022). The promoter mutants displayed approximately six-fold titer increases compared with the wild type (Fig. 2d). This finding is similar to a previous study of promoter exchange reporting up to an eightfold titer increase in promoter-swap mutants (Pistorius et al. 2022).

Discussion

Burkholderia species are prominent sources of valuable natural products (Kunakom and Eustáquio 2019) and have emerged as promising cell factories for industrial biotechnology (Adaikpoh et al. 2022). *Burkholderia* genome data highlight the vast potential of these bacteria, much of which remains unexplored (Kunakom and Eustáquio 2019). This study identifies strong constitutive promoters from *B. stagnalis* TBRC 18363, providing potential genetic tools to boost gene expression and address challenges in natural product discovery as well as low production yields in Gram-negative bacteria. As our main aim is to identify strong promoters for activating secondary metabolite production, emphasis therefore has been placed on the assessment of promoter activity at the metabolite production level rather than the transcriptional level. This decision is based on previous findings that indicated a promoter's transcriptional output may not necessarily correspond to strong protein production owing to post-transcriptional regulation (Zhao et al. 2019). Among 26 promoters tested in *E. coli* and *P. putida* heterologous hosts, p2035 and p5642 demonstrated notable strength and versatility. The successful promoter exchange experiments have illustrated the practical value of p2035 and p5642. By integrating these promoters into biosynthetic gene clusters, we significantly increased production titers of icosalide and FR900359 in *B. stagnalis* TBRC 18363 and *C. vaccinii*, respectively. These findings highlight the potential of these promoters to activate and optimize cryptic or weakly expressed pathways, which are abundant in many Gram-negative bacteria but often remain untapped due to regulatory limitations.

In this study, promoter sequences for characterization were identified by sequence location. All sequences are intergenic regions between the start codon of the selected gene and the upstream gene. Promoter sequences varied in length from 160 to 502 bp (Table S4). However, the core promoter regions were not extensively mapped. To investigate this, we employed Promotech software (Chevez-Guardado and Peña-Castillo 2021), a promoter prediction tool, to identify core promoter regions. Interestingly, in some cases, the software failed to detect core promoter regions, suggesting the presence of non-canonical promoter elements or alternative mechanisms for RNA polymerase recognition. While alternative prediction tools are available, many are tailored to specific microorganisms, which can limit their accuracy when applied to other systems. In contrast, Promotech was chosen for its broader applicability. If additional

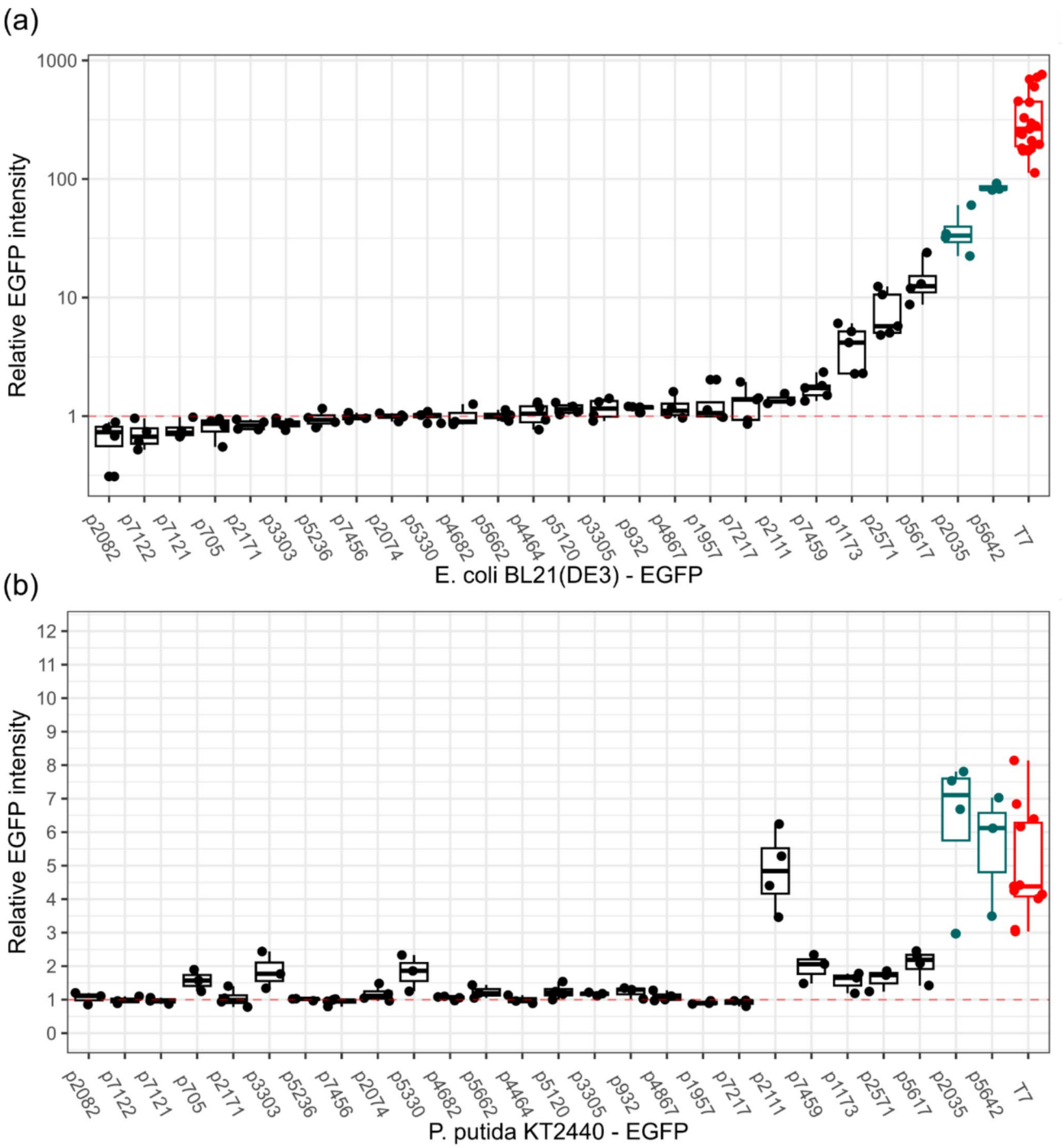

Fig. 1 *B. stagnalis* TBRC 18363 promoter activities in different reporter systems. **a** EGFP reporter in *E. coli* BL21(DE3) and **b** EGFP reporter in *P. putida* KT2440. The data show normalized EGFP intensities of the transformants carrying a test *B. stagnalis* TBRC 18363 promoter relative to a promoterless control. Three or more independent experiments were conducted for each reporter. Boxplots show median and interquartile range for each reporter. The threshold for the promoter activity is indicated by the dashed red line

functional validation data for these promoters become available, it could enable the use of modeling approaches to design and predict synthetic promoters that are both versatile and robust. Although this study successfully identified and characterized strong promoters in *B. stagnalis* TBRC 18363, several aspects of their architectural features remain unresolved and warrant further investigation.

Although the promoters p2035 and p5642 showed strong performance in the tested Gram-negative species, i.e., *E. coli*, *P. putida*, *B. stagnalis*, and *C. vaccinii*, several

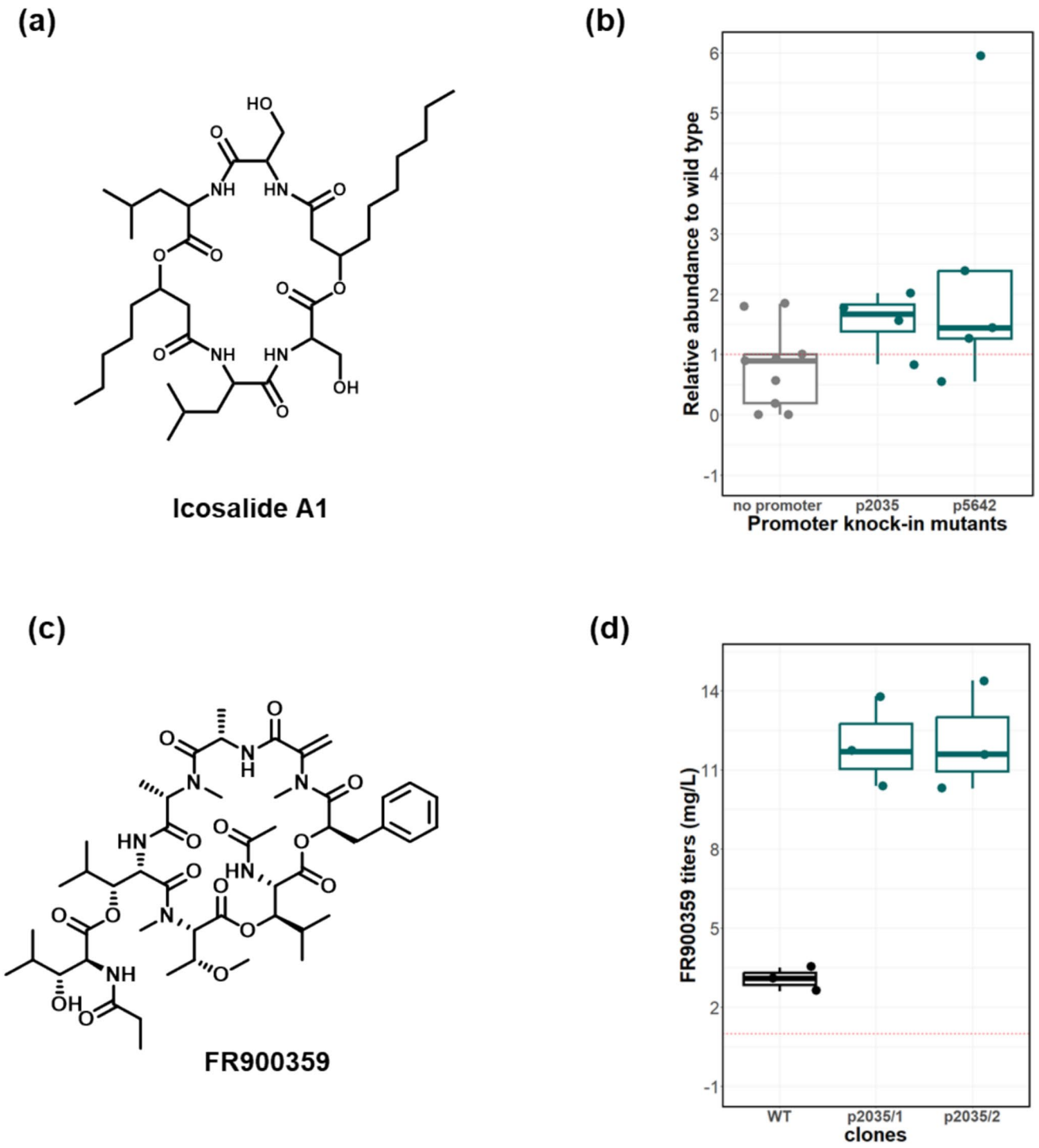

Fig. 2 **a** Structure of icosalide A1. **b** Boxplots demonstrating the relative abundance of icosalide A1 produced by the recombination control strain (no promoter), p2035, and p5642 promoter knock-in mutants relative to wild type. The recombinant control was generated by integrating the pTetCM_ICS construct lacking an added promoter at the *ics* locus using the same procedure as that for the promoter-swap mutants. Bold lines indicated the median values of the relative abundance from at least three independent experiments. **c** Structure of chromodepsin FR900359. **d** FR900359 mean titers of wild type and two p2035 mutant clones. All strains were grown in triplicate in LB medium. Boxplots show the median values and interquartile ranges

questions remain unanswered. For once, their effectiveness under different cultivation conditions has not been evaluated in depth. While these promoters have successfully enhanced the production of icosalide and FR900359, scalability remains a concern for industrial applications. Moving from lab-scale to large-scale fermentation could introduce unanticipated challenges, such as shifts in oxygen availability, nutrient limitations, or changes in cell density, which may affect promoter performance (Xiong et al. 2018). In fact, studies have shown that industrial production strains often behave differently under bioprocess conditions compared to lab-scale models (Wehrs et al. 2019). Therefore, further testing in pilot-scale fermentations is necessary to ensure that the robustness of p2035 and p5642 promoters holds up in real-world applications.

The broader implications of this work extend beyond the specific systems tested. By expanding the synthetic biology toolkit for Gram-negative bacteria, we can accelerate the discovery and sustainable production of natural products with pharmaceutical relevance. The application of p2035 and p5642, combined with other emerging tools in the field, will unlock the hidden biosynthetic potential of underexplored microbial species. This, in turn, can lead to the discovery of new drug candidates and improve the production titer of microbial-based production systems for known therapeutics. In conclusion, the p2035 and p5642 promoters represent promising genetic tools for enhancing natural product biosynthesis in Gram-negative bacteria. Based on the hosts examined in this study, these promoters exhibited cross-species functionality and can activate biosynthetic gene clusters in both *B. stagnalis* and *C. vaccinii*. While promoter performance may vary depending on the organism and genomic context, our results demonstrate their potential usefulness for metabolic engineering in selected Gram-negative bacteria. Continued exploration of these promoters and other genetic elements will further expand our capacity to harness microbial systems for drug discovery and industrial biotechnology.

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Author contribution A.A. and K.B. conceived and designed the study. All authors participated in performing the experiments and analyzing the data. C.W., W.M., T.P., U.U., P.Y., D.P., K.B., and A.A. contributed to data interpretation and manuscript preparation. V.V., U.U., P.Y., K.B., and A.A. provided significant revisions, and all authors reviewed and approved the final manuscript.

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Data availability Data is provided within the manuscript or supplementary information files.

Declarations

Ethics approval and consent to participate This article does not contain any studies with human participants or animals performed by any of the authors.

Competing interests The authors declare no competing interests.

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