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Genomic and physiological traits provide insights into ecological niche adaptations of mangrove endophytic *Streptomyces parvulus* VCCM 22513

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Abstract

Purpose: Endophytic *Streptomyces parvulus* VCCM 22513 isolated from *Bruguiera gymnorrhiza* in Quang Ninh mangrove forest, northern Vietnam showed abiotic stress tolerance consisting of antioxidant, salt-tolerant, and aromatic-compound degrading activities. The goal of this study was to shed light on genomic bases rendering mangrove endophytic *S. parvulus* more resilient to environmental stressors.

Methods: Phenotypic analysis including antioxidant activities, hydrogen peroxide and sodium chloride resistance, and aromatic compound utilization were evaluated. The genome of strain VCCM 22513 was sequenced using Illumina Miseq sequencing platform and assembled using SPAdes.

Results: Out of 15 endophytic actinomycetes associated with *B. gymnorrhiza* in Quang Ninh mangrove, northern Vietnam, VCCM 22513 extract showed remarkable antioxidant activities through (1,1-diphenyl-2-picrylhydrazyl) DPPH and superoxide radical scavenging assays of 72.1 ± 0.04% and 38.3 ± 0.16% at 1.6 mg/ml, respectively. The genome consists of a 7,688,855 bp linear chromosome, 6782 protein-coding sequences, and 68 tRNAs. Genomic analysis identified strain VCCM 22513 as *Streptomyces parvulus* and confirmed a highly conserved core genome and stability of *S. parvulus* under natural selection. Genome mining revealed the presence of genetic determinants involved in mycothiol and ergothioneine biosynthesis (26 genes), oxidative stress resistance (43 genes), osmoadaptation (87 genes), heat and cold stress (34 genes), aromatic compound degradation (55 genes). Further genome-wide comparison between *S. parvulus* VCCM 22513 and 11 *Streptomyces* genomes showed that VCCM 22513 possesses significantly higher copies of genes involved in mycothiol and ergothioneine biosynthesis. In support of this finding, the strain exhibited much resistance to 0.6–1.0 M H_2O_2 and 6% (w/v) NaCl as compared to *Streptomyces cavourensis* YBQ59 isolated from *Cinnamomum cassia* Prels. In addition, the complete pathways for degradation of aromatic compounds including protocatechuate, gentisate, 4-hydroxyphenylpyruvate, cinnamate, 3-phenylpropionate, and styrene were only identified in the genome of VCCM 22513.

Conclusions: The present study revealed for the first time adaptive responses of mangrove endophytic *S. parvulus* VCCM 22513 to survive in hostile environment. The information shown here provided better understanding of

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underlying mechanisms related to adaptation and partially plant-microbe interaction of *Streptomyces* associated with mangrove plants.

Keywords: Antioxidant, *Bruguiera gymnorrhiza*, Environmental stressors, Genome mining, Genome-wide comparison, *Streptomyces parvulus*

Introduction

Existing along the intertidal zone between terrestrial and the sea, mangroves represent distinct ecological niches, mainly found in Southeast Asia. The natural environment for mangrove plants is considered a complex set of abiotic and biotic stress factors such as salinity, oxygen deficiency, flooding, UV-B, and water logging, which trigger the excessive production of reactive oxygen species (ROS) leading to irreversible cellular damages (Ali et al. 2017; Asaeda and Barnuevo 2019). ROS are constantly generated as toxic by-products of aerobic metabolism including superoxide anion, hydroxyl radical, and hydrogen peroxide (Tan et al. 2017; Kemung et al. 2020). In this context, mangrove plants such as *Bruguiera gymnorrhiza* have developed mutualistic relationship with endophytic bacteria that provide various enzymatic and non-enzymatic antioxidants to withstand unfavorable environmental conditions (Afzal et al. 2019). Previous studies proved that the utilization of endophytic bacteria can favor host plants by contributing to salt tolerance and ROS scavenging (Alkio et al. 2005; Ali et al. 2017). Endophytic bacteria isolated from mangrove plant *Avicennia marina* were shown to neutralize oxidative burst under salinity stress by modulating antioxidants including superoxide dismutase, catalase, polyphenol oxidases, and peroxidase (Ali et al. 2017). Therefore, it is valuable to explore strategies for environmental niche adaptations of endophytic bacteria present in mangrove plants.

Actinobacteria stand out as the most ecological and biotechnological important prokaryotes. They are able to synthesize various bioactive secondary metabolites such as antiviral, antibacterial, antioxidant, and anticancer compounds, accounting for two-third of all known antibiotics (Afzal et al. 2019; Quach et al. 2021). Of these actinobacteria, members of the genus *Streptomyces* produced 80% of the total actinobacterial metabolites, leading to an increasing interest in exploitation of *Streptomyces*, especially those from terrestrial sources in the last decades (Maiti et al. 2020). However, the ability to find novel metabolites with biological activities recently reaches a stagnant point, thus attention has been shifted towards *Streptomyces* from unexplored area such as the mangrove environment. Upon adaptations to diverse environmental stressors, changes in the metabolic pathways of mangrove *Streptomyces* species might lead to the capability to synthesize diverse and novel secondary metabolites. A notable illustration of this potential in mangrove endophytic *Streptomyces* is the discovery of a novel metabolite, xiamycin A, exhibiting selective anti-HIV activity (Ding et al. 2010). Given that oxidative stress is a condition characterized by elevated amounts of free radicals contributing to cancer initiation and progression (Reuter et al. 2010), natural antioxidants produced by *Streptomyces* effectively scavenge excessive free radicals and protect cells from severe damages during oxidative burst (Law et al. 2017; Kemung et al. 2020). Most *Streptomyces* spp. capable of producing antioxidant have mainly been isolated from mangrove soils and sediments such as *Streptomyces mangrovisoli*, *Streptomyces antioxidans*, *Streptomyces colonosanans*, and *Streptomyces parvulus* (Law et al. 2017; Hu et al. 2018). This readily prompted the study of endophytic *Streptomyces* derived from mangrove plants.

To date, there are growing interests in exploring bacterial genomes to decipher the genetic bases for environmental niche adaptations and secondary metabolite biosynthesis potential. The rapid development of whole genome sequencing technology has improved data quality and analysis of the bacterial genomes, leading to an increasing number of bacterial genomes available publicly (Tian et al. 2016; Sun et al. 2018). Genome-wide comparison revealed that despite being closely-related to their terrestrial counterparts including *Streptomyces albus* J1074 and *Streptomyces flavovirens* ATCC 33331, marine sponge-associated *Streptomyces* sp. SM17 and SM18 were shown to produce different secondary metabolites and comprise 29 unique environmental niche adaptation genes (Almeida et al. 2019). For adaptation to an endophytic lifestyle, *Kitasatospora* sp. SUK42 has a reduced genome size due to the burden of encoding unnecessary metabolic pathways (Zin et al. 2021). Besides, a previous study proved that presence of genetic determinants involved in horizontal gene transfer, mobile genetic elements, and aromatic compound degradation was recognised as an adaptation mechanism of *Azoarcus* sp. CIB to its endophytic lifestyles (Martín-Moldes et al. 2015).

Quang Ninh mangrove forests locating in northern Vietnam is a rich mangrove flora consisting of 5 plant species that have been unexplored for its potent endophytes (Hanh et al. 2018; Dang et al. 2020). An earlier study using genome mining demonstrated *S. parvulus*

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03 genome derived from mangrove plant *Kandelia candel* was able to secrete melanin and desferrioxamine B (Hu et al. 2018), however environmental niche adaptation of this species remained unknown. This study aims to explore the phylogenomic positions of *S. parvulus* VCCM 22513 and shed light on the genetic determinants rendering it more resilient to model environmental stressors (salt, H_2O_2, and aromatic compounds). This is the first report deciphering underlying mechanisms related to multiple stress resistance and aromatic catabolism of mangrove endophytic *S. parvulus* VCCM 22513 at the genomic and phenotypic level.

Materials and methods

Isolation and preservation of endophytic actinomycetes

Actinomycetes were isolated from healthy mangrove plant *Bruguiera gymnorrhiza* collected at Quang Ninh mangrove forest, Northern Vietnam (21.0064° N, 107.2925° E). After sampling, the roots, stems, and leaves were placed in sterile plastic bags, transported to the laboratory within 48 h. The samples were washed with running tap water and distilled water. To eliminate unwanted microorganisms, surface sterilization procedure was carried out as described previously (Vu et al. 2020). In brief, the leaves, stems and roots were cut into small segments (1–2 cm) followed by surface sterilization via successive immersion into 15% (v/v) NaClO for 5 min, 2.0% (w/v) $Na_2S_2O_3$ for 2 min, washed three times with sterile water. After that, the resulting samples were treated with 70% ethanol for 7 min, washed three times with sterile water, then dried under laminar flow conditions. After surface sterilization, the samples were frozen at − 80 °C for 2 weeks, and then spread onto 8 selective isolation media including humic acid-vitamin B, raffinose-histidine, tap water-yeast extract, International Streptomyces Project 5 (ISP5), trehalose-proline, sodium succinate-asparagine, starch, and citric acid agars supplemented with 50 mg/ml nystatin, 25 mg/ml $K_2Cr_2O_7$, and 25 mg/ml nalidixic acid as described previously (Musa et al. 2020; Vu et al. 2020). Once observed, actinomycete colonies were streaked out several times on International *Streptomyces* Project (ISP) 2 medium to get pure isolates (Shirling and Gottlieb 1966). The pure cultures were stored in 20% glycerol at − 80 °C.

The 16S rRNA gene of each isolate was amplified using the universal primers 27F and 1492R as described previously (Vu et al. 2020). The resulting PCR products were sent to First BASE Laboratories Sdn. Bhd. (Malaysia) for Sanger sequencing. The obtained sequences were trimmed and compared with representative 16S rRNA gene sequences of related type strains retrieved from the GenBank database (NCBI).

Screening of antioxidant activities

All isolates were inoculated on YIM38 medium (glucose 4 g; yeast extract powder 4 g; malt extract powder 5 g; thiamine-HCl, riboflavin, niacin, pyridoxine-HCl, inositol, calcium pantothenate, and *p*-aminoben-zoic acid, each 0.5 mg; biotin 0.25 mg; pH 7.0 per liter) for 10 days at 30 °C with shaking at 200rpm (Nguyen et al. 2019). The biomass was removed by centrifugation while the supernatant was subjected to freeze-drying process. Extraction of freeze-dried samples was carried out using absolute methanol that underwent subsequent evaporation at 40 °C (Tan et al. 2017). The resulting extract was dissolved in dimethyl sulfoxide (DMSO) for antioxidant assays.

In the superoxide radical scavenging assay, 4-ml mixtures containing 20 μM phenazine methosulfate, 160 μM NADH, 55 μM nitro blue tetrazolium (NBT), the extract (0.2–1.6 mg/ml), and 0.2 M sodium phosphate buffer (pH 7.3) were incubated at room temperature for 10 min. Superoxide radical scavenging activity was calculated based on the absorbance measured at 560 nm as reported previously (Rajoka et al. 2019). For the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, about 0.2 ml of extract solution (0.2; 0.4; 0.8; 1.6 mg/ml) in methanol was reacted with 0.2 ml of 0.1 mM DPPH solution followed by the addition of 2.0 ml of deionized water. The reaction was incubated in the dark for 30 min at room temperature. DPPH radical scavenging activity of VCCM 22513 extract was determined using absorbance measured at 517 nm as previously described (Liu et al. 2010; Vu et al. 2021). In the reducing power assay, the mixtures of 1.5 ml of the crude extract (0.2, 0.4, 0.8, 1.6 mg/ml), 1.5 ml of 0.2 M sodium phosphate buffer (pH 7.3), and 1.5 ml of 1% (w/v) $K_3Fe(CN)_6$ were incubated at 50 °C for 25 min. The reactions were terminated by adding 1.5 ml of 12% (w/v) trichloroacetic acid, followed by centrifugation. The supernatants were reacted with 0.5 ml of 0.2% (w/v) $FeCl_3$ and absorbance was measured at 700 nm (Rajoka et al. 2019). Ascorbic acid was used as the positive control for all assays.

Morphological and physiological characteristics of VCCM 22513

The bioactive isolate VCCM 22513 was cultivated on ISP2-7 agar media at 30 °C for 10 days to evaluate morphological characteristics such as aerial and substrate mycelium color as well as pigment production as described previously (Quach et al. 2021). Microscopic cell morphology was observed using a conventional microscope (Carl Zeiss) with 100× oil immersion objective. Physiological properties of VCCM 22513 were assessed under different conditions of pH (2.0-9.0) and temperature (16-42°C). The utilization of different carbon sources

(1%, w/v) was examined on ISP9 medium. In addition, the ability to produce exoenzymes such as amylase, cellulase, chitinase, protease, and xylanase was determined following previous protocol (Kemung et al. 2020).

Growth assessment of VCCM 22513 in response to H_2O_2, NaCl, and aromatic compounds

In the H_2O_2 tolerance test, the strain VCCM 22513 was grown until exponential phase and then spread on ISP-2 agar plates. Sterile paper disks soaked in 10 μl of hydrogen peroxide (H_2O_2; 0.6, 0.8, 1.0, and 2.0 M) were deposited on the prepared plates (Undabarrena et al. 2017). Growth inhibition diameters were determined after 4 days of incubation at 30 °C. For the NaCl tolerance test, the strain VCCM 22513 was plated on ISP-2 agar plates with 0, 2, 4, and 6% (w/v) NaCl (Zin et al. 2021). Bacterial growth was monitored after 16 days of incubation at 30 °C. For aromatic compound degradation experiments, the strain VCCM 22513 was inoculated in CM basal medium (L-asparagine 0.5 g; K_2HPO_4 0.5 g; $MgSO_4 \cdot 7H_2O$ 0.2 g; $FeSO_4 \cdot 7H_2O$ 0.01 g; casein hydrolysate 0.05 g; pH 7.0 per liter) supplemented with gentisate (60 μM), cinnamate (80 μM), styrene (80 μM), or protocatechuate (60 μM) (Khalil et al. 2019). The CM basal medium without aromatic compounds was used as a negative control. The growth of strain VCCM 22513 was evaluated after 8 days at 30 °C based on the comparison to the control. All experiments were performed in triplicate.

Genome sequencing, assembly, and annotation of the *S. parvulus* VCCM 22513

Genomic DNA of strain VCCM 22513 was extracted using G-spin™ Total DNA Extraction Mini Kit (Intron Bio, Korea) according to the manufacturer's protocol. Whole genome of VCCM 22513 was sequenced with Illumina Miseq sequencing platform (Illumina, California, USA). Quality control and read trimming were performed by FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and Trimmomatic 3.0, respectively (Bolger et al. 2014). De novo assembly with default parameters and k-mer = 21, 33, 55, 77 was performed using SPAdes 3.15 (Bankevich et al. 2012). Benchmarking Universal Single-Copy Orthologous (BUSCO) 3 (https://gitlab.com/ezlab/busco) was used to evaluate the completeness of the assembled genome. Genome features were analyzed using 2 pipelines including Prokka v1.12 and Prokaryotic Genomes Annotation Pipeline (PGAP) at NCBI (Seemann 2014, Tatusova et al. 2016). Functional annotation of the predicted coding genes was performed based on EggNOG database (Huerta-Cepas et al. 2019). Default parameters were applied for all software tools.

Genome-wide comparison

The assembled sequence of VCCM 22513 was uploaded to Type Strain Genome Server TYGS server (https://tygs.dsmz.de) for whole genome-based taxonomic analysis. Taxogenomic analyses with closely related species were implemented by calculating the overall genome-related indexes including digital DNA–DNA hybridization (dDDH), G + C difference, and distance formula d5. The 16S rRNA gene sequence was retrieved from VCCM 22513 genome and aligned with related type *Streptomyces* strains accessible on the GenBank databases using CLUSTAL-X software. Sequence similarities were calculated based on the EzBiocloud database (Kim et al. 2012). The phylogenetic tree was built by the neighbor-joining method using Molecular Evolutionary Genetics Analysis (MEGA) software version 7 with Kimura-2-parameter distances (Kumar et al. 2016). *Streptomyces inhibens* NEAU-D10[T] was used as an outgroup branch.

The genome of VCCM 22513 was compared to 11 *Streptomyces* strains available on GenBank: *S. parvulus* 2297 (CP015866); *S. parvulus* JCM 4068[T] (BMRX00000000); *S. parvulus* LP03 (JAIWPL000000000); *Streptomyces cavourensis* YBQ59 (QLNH00000000); *Streptomyces venezuelae* ATCC 10712[T] (CP029197); *Streptomyces albus* J1074 (CP004370); *Streptomyces flavovirens* ATCC 33331 (CP002475), *Streptomyces olivaceus* B-3009[T] (JADOEJ000000000), *Streptomyces gilvigriseus* MUSC26[T] (MLCF00000000), *Streptomyces mangrovisoli* 149[T] (LAVA00000000), *Streptomyces monashensis* 1J[T] (MLYO00000000), *Streptomyces endocoffecius* CA3R110[T] (JAERRG000000000), *Streptomyces kebangsaanensis* SUK12[T] (JUJA00000000). Genes organized into sets of logically related functional roles were clarified based on the SEED-viewer analysis of genome sequences by RAS-Ttk (Brettin et al. 2015). The predicted protein sequences were used to predict the orthologous gene clusters among *S. parvulus* strains using OrthoVenn2 web server (Xu et al. 2019).

Identification of genes involved in adaptive mechanisms and accession number

Secondary metabolite biosynthetic gene clusters (BGCs) of VCCM 22513 were predicted by antiSMASH 5.1.2 with default parameters (Blin et al. 2021). Potential adaptation mechanisms of strain VCCM 22513 were determined via an extensive literature search for mechanisms involved in redox balance, oxidative stress response, osmoadaptation, heat and cold response, and aromatic compounds degradation that have been demonstrated in microorganisms, especially *Streptomyces*. Genes encoding for proteins of interest were predicted using the KEGG, RAST, Mapper tools and annotated using

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the BLAST tool of NCBI, GenBank database, and Conserved Domain Database (CDD). The questionable open reading frames were searched through BLASTp and TBLASTN tools (*e*-value cut-off = 1e−5) against a database of enzymes, transcriptional regulators reported in UniProtKB-Swissprot (Camacho et al. 2009; Brettin et al. 2015). An e-value cutoff < 10^{-10}, identity > 25% and coverage > 50% were used to filter the outcome.

The 16S rRNA gene and draft genome of *S. parvulus* VCCM 22513 were deposited in GenBank under accession number ON026158 and JAJVLA000000000, respectively.

Results

Isolation and phenotypic characterization of VCCM 22513

Of the 15 endophytic actinomycetes associated with mangrove plant *B. gymnorrhiza* in Northern Vietnam, isolate MX9 showed the strongest antioxidant activities (Table S1). Indeed, MX9 extract displayed significant antioxidant activity from 10.9 ± 0.12% to 72.1 ± 0.04% of DPPH radical reduction at doses ranging from 0.2 to 1.6 mg/ml, which could be due to its hydrogen donating ability (Fig. 1A). The antioxidant activity of the extract against superoxide radical was 38.3 ± 0.16% at 1.6 mg/ml, while the reducing power was observed at a low level (Fig. 1B). The strain grew well on all ISP agar and produced whitish-grey aerial mycelium and pale-yellow substrate mycelium after 6 days at 30 °C (Table S2). Physiological analysis revealed that the growth was observed at 16–42 °C (optimum 30 °C) and at pH 2.0–9.0 (optimum pH 6.0). The cells were able to produce broad range of extracellular enzymes such as amylase, cellulase, xylanase, chitinase, protease, and grow on media supplemented with gentisate, cinnamate, styrene, and protocatechuate (Table S2). The strain MX9 was then renamed as *Streptomyces* sp. VCCM 22513 for further investigation based on the

deposition at VAST-Culture Collection of Microorganisms (VCCM, www.vccm.vast.vn).

Genome sequence and general features of the *S. parvulus* VCCM 22513

Using Illumina platform, a total of 5,283,212 paired-end reads were obtained and then assembled in 62 contigs with N50 of 246,939 bp. The total size of assembled genome was 7,688,855 bp with 72.1% G + C encoding 6,782 protein-coding sequences (CDSs) and 68 tRNAs (Table S3). *Streptomyces* chromosomes vary from 6.28 Mb for *Streptomyces cattleya* NRRL 8057[T] to 11.94 Mb for *Streptomyces bingchenggensis* BCW-1. Among them, the genome size of *Streptomyces* sp. VCCM 22513 felt in the middle size range of *Streptomyces* and was quite comparable to three selected *S. parvulus* genomes (Fig. 2A).

To identify VCCM 22513 at species level, the genome-to-genome distance calculator web server was used to calculate the dDDH between VCCM 22513 genome and *Streptomyces* genomes. *Streptomyces* sp. VCCM 22513 was found to display the highest similarity to *S. parvulus* JCM 4068[T] represented by 94.7% identity dDDH and 0.02% G + C difference values (Fig. 2B). Furthermore, strain VCCM 22513 exhibited 16S rRNA gene sequence similarity values of 100% and 99.9% with *S. parvulus* NBRC 13193[T] and *S. parvulus* JCM 4068[T], respectively. Phylogenetic analysis showed that the strain VCCM 22513 only formed a well-delineated subclade with *S. parvulus* species (Fig. S1). Taken together, the strain was identified as *S. parvulus* VCCM 22513.

With the aim to study the functional categories of 6,782 coding sequences of *S. parvulus* VCCM 22513, this strain and 3 other *S. parvulus* including 2297, JCM 4068[T] from soils and LP03 from lichen were subjected to SEED using RASTtk where genes were organized into sets of logically related functional roles (Overbeek et al. 2005). The three most abundant categories in *S. parvulus*
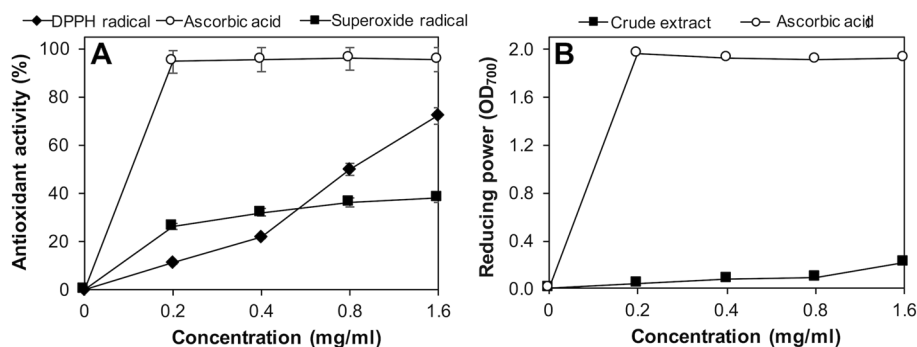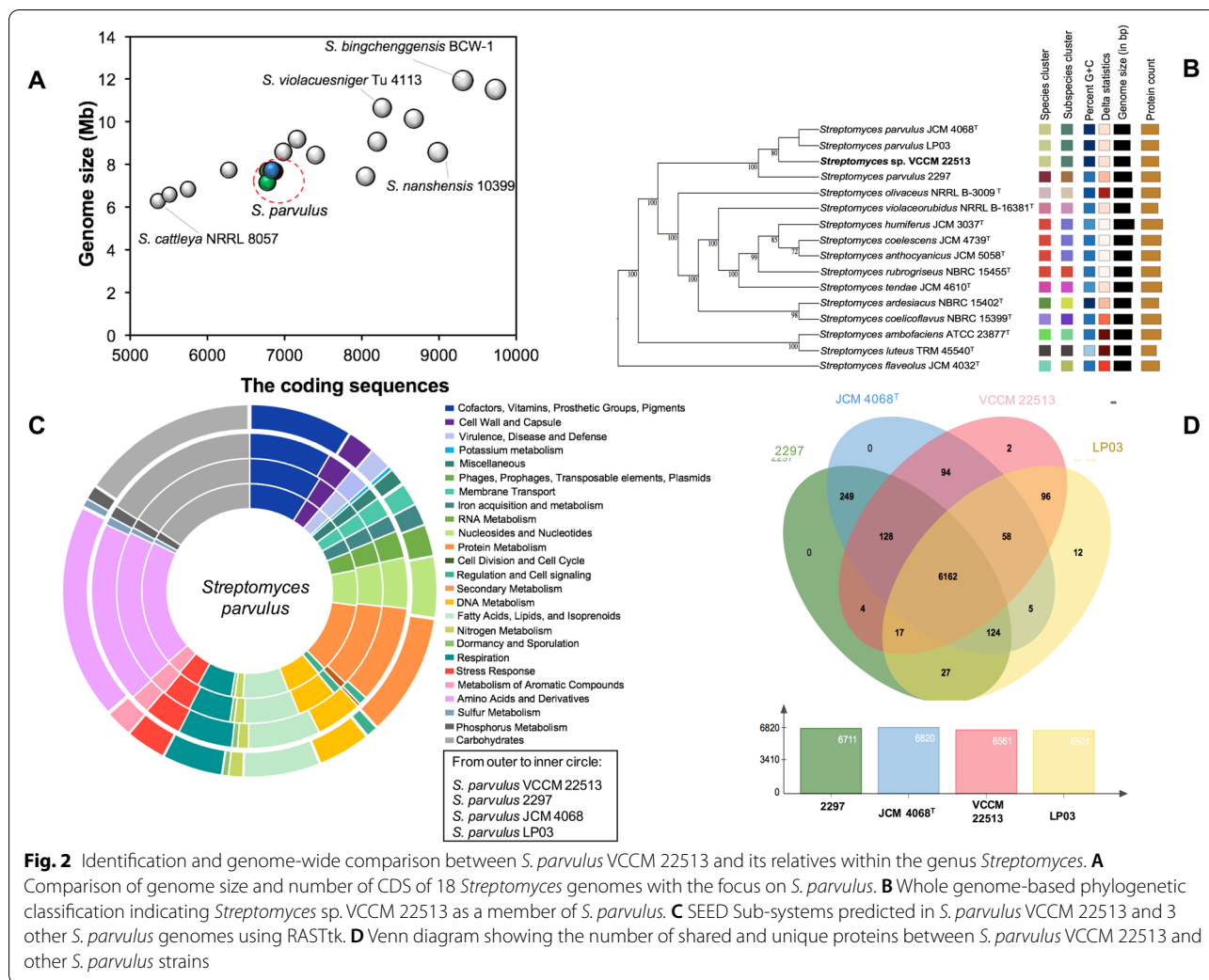

Fig. 1 Antioxidant activities including DPPH and superoxide radical scavenging activities (**A**) and reducing power (**B**) determined in the VCCM 22513 extract

Fig. 2 Identification and genome-wide comparison between *S. parvulus* VCCM 22513 and its relatives within the genus *Streptomyces*. **A** Comparison of genome size and number of CDS of 18 *Streptomyces* genomes with the focus on *S. parvulus*. **B** Whole genome-based phylogenetic classification indicating *Streptomyces* sp. VCCM 22513 as a member of *S. parvulus*. **C** SEED Sub-systems predicted in *S. parvulus* VCCM 22513 and 3 other *S. parvulus* genomes using RASTtk. **D** Venn diagram showing the number of shared and unique proteins between *S. parvulus* VCCM 22513 and other *S. parvulus* strains
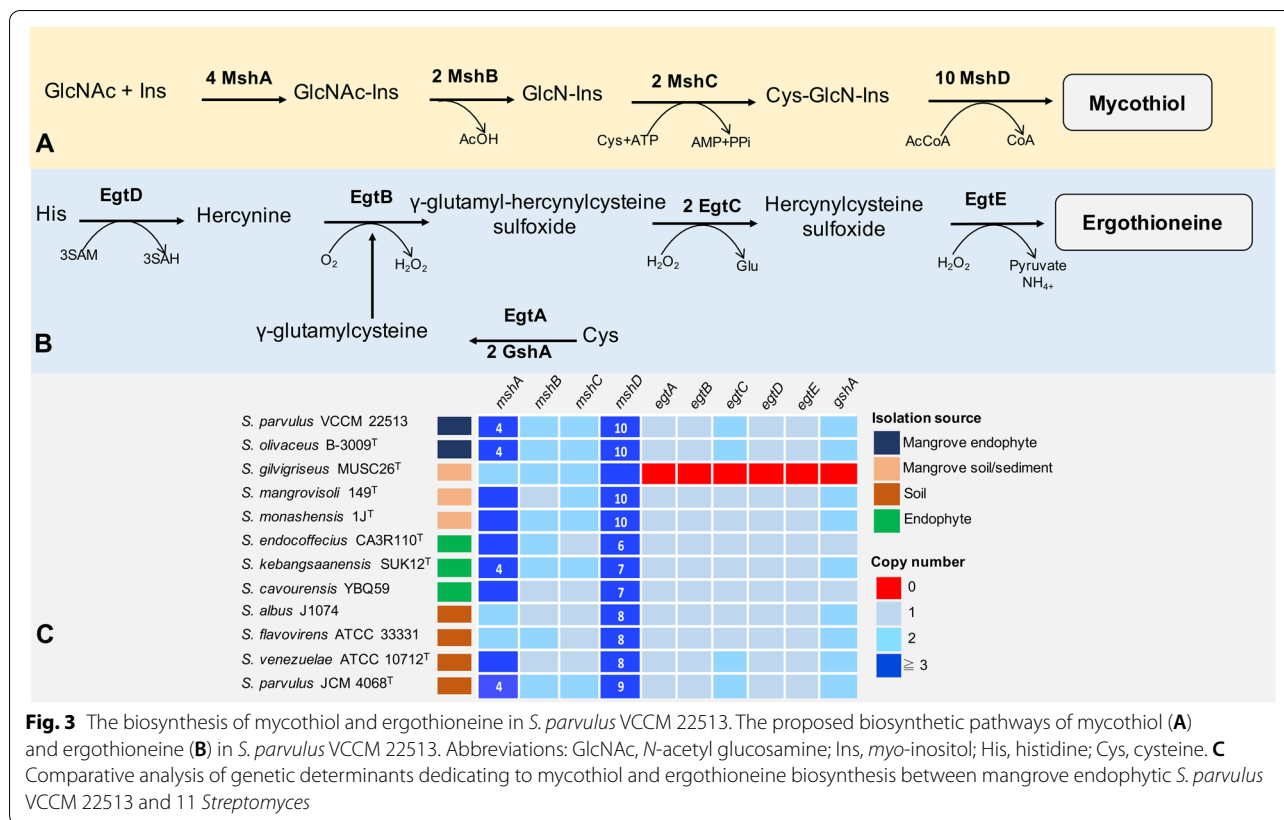
VCCM 22513 genome were amino acids and derivatives (396 genes), carbohydrates (323 genes), and protein metabolism (225 genes). Of note, VCCM 22513 encoded 97.7% of the average number of subsystems per *S. parvulus* genome (Fig. 2C). Subsystems involved in Nitrogen Metabolism (101.3%) and Metabolism of Aromatic Compounds (105.6%) were over-represented with respect to the average. The distribution of subsystems among *S. parvulus* species is highly similar implying that isolation source does not lead to the loss of the genetic materials. In support of this result, all four species shared a large core-genome of 6162 COGs and only 2 unique COGs annotated as putative proteins were found in the VCCM 22513 genome using OrthoVenn (Fig. 2D).

Maintenance of redox balance

Low-molecular weight (LMW) thiols are required for maintaining the reduced cytoplasm of bacteria, which further protect bacteria from the hostile oxidative

environments (Tung et al. 2018). In the VCCM 22513 genome, 18 genes involved in the biosynthesis of low-molecular weight thiols such as mycothiol were identified (Table S4). In the first step, the glycosyltransferase MshA (*orf_549, orf_4674, orf_5830, orf_6700*) conjugates *myo*-inositol and *N*-acetyl glucosamine to *N*-acetyl glucosamine *myo*-inositol, followed by deacetylation by metal-dependent deacetylase MshB (*orf_1123, orf_6108*) to yield glucosamine inositol (Fig. 3A). In the third step, Cys ligase MshC (*orf_1437, orf_1645*) conjugates Cys to produce Cys-GlcN-Ins. The final step involved acetylation of the Cys amino group by 10 copies of mycothiol acetyltransferase MshD to generate mycothiol. Similar to mycothiol, the complete biosynthetic pathway of ergothioneine required the presence of 5 main genes including *egtA, egtB, egtC, egtD, egtE* which were found in the VCCM 22513 genome (Fig. 3B). The four genes *egtA, egtB, egtC,* and *egtD* are clustered in an operon, and *egtE* is

Fig. 3 The biosynthesis of mycothiol and ergothioneine in *S. parvulus* VCCM 22513. The proposed biosynthetic pathways of mycothiol (**A**) and ergothioneine (**B**) in *S. parvulus* VCCM 22513. Abbreviations: GlcNAc, *N*-acetyl glucosamine; Ins, *myo*-inositol; His, histidine; Cys, cysteine. **C** Comparative analysis of genetic determinants dedicating to mycothiol and ergothioneine biosynthesis between mangrove endophytic *S. parvulus* VCCM 22513 and 11 *Streptomyces*

separately encoded in a distant locus, which are different to *Mycobacterium smegmatis* MC^2 155 and *S. coelicolor* A3(2) (Seebeck 2010; Nakajima et al. 2015). Surprisingly, *S. parvulus* VCCM 22513 also contained two copies of *gshA* (*orf_1595*, *orf_4097*) encoding glutamate-cysteine ligases that might promote ergothioneine biosynthesis through the production of precursor γ-glutamylcysteine.

Comparative genomic analysis of VCCM 22513 with 11 mangrove-, plant- and soil-derived *Streptomyces* spp. indicated that genetic determinants related to the biosynthesis of low-molecular weight thiols are present in all genomes but with a different number of copies (Fig. 3C). Despite sharing a highly conserved core genome, *mshD* genes in VCCM 22513 (10 copies) was higher than *S. parvulus* JCM 4068^T (9 copies) isolated from soil. Gene *egtC* was duplicated in the VCCM 22513 genome, which was only similar to *S. olivaceus* B-3009^T, *S. venezuelae* ATCC 10712^T, and *S. parvulus* JCM 4068^T. Of note, *S. parvulus* VCCM 22513 and *S. olivaceus* B-3009^T recovered from mangrove plant *Kandelia candel* (L.) Druce showed a similar profile of genes involved in mycothiol and ergothioneine biosynthesis, which were particularly high in gene copies as compared to 10 mangrove and terrestrial genomes selected. Interestingly, mangrove soil-derived *S.*

gilvigriseus MUSC26^T lacked the redox protective ergothioneine (Fig. 3C).

Primary responses of *S. parvulus* VCCM 22513 to oxidative stress

Given that the presence of oxygen, cycles of inundation, salinity fluctuations, and aromatic compounds can indirectly cause oxidative stress such as ROS and reactive electrophilic species (RES) to endophytes (Ali et al. 2017; Asaeda and Barnuevo 2019), mechanisms to prevent cellular damages might be important components of mangrove endophytic *S. parvulus* VCCM 22513. Investigation of genes involved in primary oxidative stress response resulted in the prediction of 43 corresponding genes (Fig. 4A, Table S5). Under oxidative stress, mycothiol is oxidized to mycothiol disulfide, which is recycled by the mycothiol disulfide reductase Mtr (*orf_5308*). Parallelly, mycothiol forms mixed disulfides with redox proteins to protect them from irreversible over-oxidation, which subsequently is catalyzed by mycoredoxin Mrx1 (*orf_1066*) to release reduced proteins. In addition, thioredoxin system found in the VCCM 22513 genome was encoded by 10 putative thioredoxin and thioredoxin reductase genes which play an important role in reducing disulfide bonds of cysteine-containing proteins.

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Fig. 4 Functional analysis of oxidative and osmotic stress tolerance of *S. parvulus* VCCM 22513 at genomic and phenotypic levels. Comparative analysis of genes involved in oxidative stress resistance (**A**) and osmotic stress (**C**) present in *S. parvulus* VCCM 22513 and 11 Streptomyces genomes. Functional response of *S. parvulus* VCCM 22513 and *S. cavourensis* YBQ59 to increasing concentrations of hydrogen peroxide (H₂O₂) (**B**) and 0–6% (w/v) NaCl (**D**)

Furthermore, genes categorized as superoxide dismutase (2 genes), alkyl hydroperoxide reductase (7 genes), peroxidase and peroxiredoxin (6 genes), catalase (4 genes), and ROS resistance protein (5 genes) were predicted, in which several are duplicated and multiplicated (Fig. 4A). In line with these results, global oxidative stress responses of strain VCCM 22513 might be regulated by 6 universal transcription factors such as redox-sensitive transcriptional regulator SoxR, regulatory protein SoxS, hydrogen peroxide-sensing transcriptional regulator OxyR, peroxide-responsive repressor PerR, organic hydroperoxide resistance transcriptional regulator OhrR, and ferric uptake regulator FurA (Table S5). Further comparative genomics showed that the number of genes encoding for enzymatic scavengers including ROS resistance protein, redox transcriptional regulator, catalase, peroxidase, and peroxiredoxin found in *S. parvulus* VCCM 22513 were similar to those in the genomes of terrestrial and mangrove *Streptomyces* strains (Fig. 4A). The differences were that *S. parvulus* VCCM 22513 possessed an extra copy

of alkyl hydroperoxide reductase *ahp* and bromoperoxidase-catalase *bca* in comparison to other mangrove genomes including *S. olivaceus* B-3009ᵀ, *S. gilvigriseus* MUSC26ᵀ, *S. mangrovisoli* 149ᵀ, and *S. monashensis* 1Jᵀ.

Besides enzymatic scavengers, *S. parvulus* VCCM 22513 also utilizes secondary metabolites responsible for neutralizing oxidative damages. Using AntiSMASH, in silico prediction of secondary metabolite biosynthetic gene clusters identified a total of 25 clusters with similarity to known clusters of ≥ 16%. Of those, 4 biosynthetic gene clusters including desferrioxamin, melanin, coelichelin, and isorenieratene were reported to be antioxidant and iron chelating agents (Table S6) (Hu et al. 2018; Chen et al. 2019; Williams et al. 2019; Wibowo et al. 2022). This was in agreement with the DPPH, reducing power, superoxide radical scavenging activities observed, indicating the ability of strain VCCM 22513 to produce 4 antioxidant compounds.

To correlate with the genetic content of redox balance and oxidative stress properties, the response of

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strain VCCM 22513 to H_2O_2 concentrations was tested and compared to *S. cavourensis* YBQ59 from *Cinnamomum cassia* Prels (Tung et al. 2018). Strain VCCM 22513 showed smaller growth inhibition zones in response to 0.6, 0.8, and 1.0 M H_2O_2 as compared to *S. cavourensis* YBQ59 (Fig. 4B), indicating great capability of mangrove endophytic VCCM 22513 in neutralizing H_2O_2 toxicity. At 2.0 M H_2O_2, no significant difference was observed (Fig. S2).

Distribution of genes involved in osmoadaptation

To protect themselves from variations in external osmotic pressure, actinomycetes have to accumulate and release solutes by regulating water fluxes and maintaining cellular homeostasis (Yaakop et al. 2016; Undabarrena et al. 2017). In the present study, 87 genes involved in the defensive responses to osmotic stress were identified in the VCCM 22513 genome (Fig. 4C). Given that proton pump is crucial to produce ATP via proton-motive force and to maintain a proton gradient under high-salt stress (Sun et al. 2018), NADH quinone oxidoreductase genes such as *nuo* and *ndh* encoding proton pumps were found in 2 operon-like structures including *nuo* (14 genes) and *ndhC-nuoN* (10 genes), making them the most abundant groups identified in the genome (Table S7). In contrast, the partial *nuo* operon (8 genes) was present in the genome of marine actinomycetes such as *Streptomyces* sp. SM17 and *Streptomyces* sp. SM18 (Almeida et al. 2019).

Additionally, *S. parvulus* VCCM 22513 might also be able to synthesize ectoine and its derivative, 5-hydroxyectoine, as compatible solutes to maintain the osmotic equilibrium in response to salt stress. In line with having the *ectABCD* operon, transporters for ectoine encoded by *ehuABCD* were revealed, supporting the ability to tolerate saline stress of the mangrove endophytic strain VCCM 22513 (Table S7). Conversely, in a low-salt stress scenario, biosynthesis and uptake of compatible solutes including proline (15 genes), glutamate (17 genes), glutamine (14 genes), and glycine betaine/choline (8 genes) are likely employed as another essential defense mechanism of VCCM 22513. In halophilic bacteria, proline is thought to be the abundant compatible solute upon increasing osmotic stress. Meanwhile, the proline/betaine transporter ProP is not specific to its substrates leading to its versatility to transport various solutes such as proline, glycine betaine, proline betaine, ectoine, and other compounds (Saum and Müller 2007; Burg and Ferraris 2008).

Comparative genomics analysis revealed that 5 copies of glutamine transport ATP-binding *glnQ* related to glutamine synthesis were determined in 3 soil-derived genomes, while only 3–4 copies were present in the mangrove genomes (Fig. 4C). For glutamate synthesis, the

genetic determinants found in the VCCM 22513 genome were quite comparable with mangrove endophytic *S. olivaceus* B-3009[T], except for *gdhI* encoding dehydrogenase reductase. It is worthy to note that this gene was present in the terrestrial genomes, but not in the mangrove-derived genomes. In addition, further analysis revealed 5 copies of a potential proline/betaine transporter *proP* in the VCCM 22513 genome, unlike terrestrial genomes such as *S. albus* J1074, *S. flavovirens* ATCC 33331, and *S. venezuelae* ATCC 10712[T] in which *proP* exists in only three copies (Fig. 4C). In support of these genetic findings and to explore the potential to resist osmotic stress, the strain VCCM 22513 and *S. cavourensis* YBQ59 were plated on ISP-2 agar supplemented with 0–6% NaCl (w/v). The presence of 2% NaCl did not affect the growth and sporulation of strains VCCM 22513 and YBQ59. However, exposure to 6% NaCl resulted in a significant growth delay of YBQ59, but not VCCM 22513 after 16 days (Fig. 4D). These results indicated that mangrove endophytic VCCM 22513 was notably more tolerant to NaCl than the terrestrial endophytic YBQ59.

Mechanisms of dealing with heat and cold stress

Despite being protected by the host *B. gymnorrhiza*, mangrove endophytic *S. parvulus* VCCM 22513 might equip with the genetic determinants to adapt to sudden temperature shift. Genomic analysis confirmed the presence of 34 predicted genes may play a role in such tolerance (Table S8). For heat shock response, the *dnaK* operon containing *dnaK*, *grpE*, *dnaJ*, and *hspR* was identified, which is conserved across Actinobacteria such as *S. coelicolor*, *Corynebacterium glutamicum*, and *Mycobacterium bovis* (Segal and Ron 1996; Ventura et al. 2005). Heat shock repressor HrcA may regulate the expression of *dnaJ*, *clpB*, *groSL*, *grpE*, *htpG* and itself upon exposure to changes in temperatures. The predicted proteases include 2 Lon (cytoplasmic), 3 HtpX (membrane), and 1 DegP (periplasmic) which play a vital part in eliminating protein aggregates or damaged proteins as a consequence of heat shock. Regarding cold-induced stress, 3 major cold-shock proteins Csp, Cps, and ScoF are present in the VCCM 22513 genome (Table S8). In addition, the cells can also be protected by transcription termination proteins NusAB and DEAD-box ATP-dependent RNA helicase CshA that are expressed during temperature downshift. These results are in agreement with the wide range of temperatures in Northern Vietnam which varies from 5 to 38 °C.

Prediction of metabolic pathways for aromatic compound utilization

Being able to resist aromatic compound stress is important in helping bacteria survive within host plants

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(Martín-Moldes et al. 2015; de Melo et al. 2018). Phenotypic analysis displayed that *S. parvulus* VCCM 22513 was able to grow in the presence of gentisate, cinnamate, styrene, and protocatechuate (Fig. S3). In agreement with these results, genome mining revealed the gene cluster encoding protocatechuate meta-cleavage pathway distributed along the chromosome of VCCM 22513, including β-ketoadipate succinyl-CoA transferase subunit α and β (*pcaI, pcaJ*), protocatechuate 3,4-dioxygenase subunit α and β (*pcaH, pcaG*), 3-carboxy-cis,cis-muconate cycloisomerase (*pcaB*), muconolactone D-isomerase (*catC*) and 3-oxoadipate enol-lactonase (*catD*), and 2 regulatory proteins (*nodD, hosA*) (Fig. 5A). It should be noted that the gene *catD* encoding 3-oxoadipate enol-lactonase that converts β-ketoadipate-enol-lactone to β-ketoadipate was found in 4 copies along the genome of VCCM 22513 and terrestrial endophytic *S. endocoffecius* CA3R110[T] and *S. kebangsaanensis* SUK12[T]. Surprisingly, all reference genomes contained incomplete protocatechuate degradation pathway due to the lack of some important genes such as *catC, pcaHG, pacB,* and *pcaR* (Fig. 5B).

In contrast to protocatechuate, genes involved in gentisate catabolism are not clustered in an operon. The identified gentisate pathway consists of 4 main genes including transcriptional regulator (*kdgR*), gentisate 1,2-dioxygenase (*sdgD*), fumarylacetoacetate hydrolase (*nagK*), and maleylpyruvate isomerase (*nagL*) (Table S9). The SdgD is a key component in oxygenating the benzen ring of gentisate to form 3-maleylpyruvate, which is further metabolized by 6 NagL and 4 NagK enzymes into TCA cycle intermediates such as fumarate and pyruvate. Additionally, the homogentisic pathway, a central pathway for catabolism of phenylalanine and tyrosine, was identified with 4-hydroxyphenylpyruvate as the precursor

of homogentisic acid which is converted into the final product maleacetoacetate by 4-hydroxyphenylpyruvate dioxygenases (*hpd*) and homogentisate 1,2-dioxygenase (*hmgA*).

Furthermore, genome annotation showed 15 genes related to peripheral pathways which diverged into the styrene upper and lower catabolic pathways (Fig. 5A). Moreover, *mhp* and *hca* genes encoding the putative peripheral pathways for the degradation of 3-phenylpropionate as well as cinnamate were found distributed along the VCCM 22513 genome. The styrene upper catabolic pathway consisted of *styA* and *styD* genes, leading to formation of phenylacetate which is then converted into metabolites needed for the TCA cycle by proteins encoded by *paaABCDEFGHIJKX* genes present in the styrene lower catabolic pathway (Table S9). Out of 12 compared genomes, the complete styrene degradation pathway was only found in the genome of strains VCCM 22513, B-3009[T], 149[T], and JCM 4068[T] (Fig. 5B). Different to the protocatechuate and styrene pathways, catabolic pathways for gentisate, 4-hydroxyphenylpyruvate, 3-phenylpropionate, and cinnamate were reported to be complete in most compared genomes.

Discussion

The mangroves are a unique ecosystem and characterized by the presence of a few plant species such as *B. gymnorrhiza* that is adapted to higher-than-normal levels of salinity, fluctuations in tidal gradients, temperature and other oxidative stresses. To survive in harsh environments, mangrove endophytic *Streptomyces* might be well-equipped with a number of genetic determinants encoding enzymatic, non-enzymatic, and secondary

Fig. 5 Complete pathways of aromatic compound degradation identified in *S. parvulus* VCCM 22513. **A** Six peripheral catabolic pathways for the aromatic compound degradation and their corresponding metabolites predicted in the *S. parvulus* VCCM 22513 genome. Ring-cleavage product structures of central aromatic catabolic pathways are shown inside the circle, which are fueled into tricarboxylic acid (TCA) cycle. **B** Distribution of genes involved aromatic compound degradation in *S. parvulus* VCCM 22513 and 11 *Streptomyces* strains

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metabolite agents (Tan et al. 2017; Jiang et al. 2018). In this study, a robust adaptive response to environmental stressors of *S. parvulus* VCCM 22513 associated with *B. gymnorrhiza* was deciphered at the phenotypic and genetic levels to provide better understanding of adaptation of actinobacteria to mangrove endophytic lifestyle.

In natural habitats, actinobacteria have evolved to adapt to the environmental niches and compete with multispecies communities for resources and space, leading to metabolic and physiological differences (Almeida et al. 2019; Zerouki et al. 2021; Zin et al. 2021). For example, the growth of marine sponge-derived *S. albus* SM17 was improved in high salinity medium as opposed to soil-derived *S. albus* J1074, mainly due to a pool of 29 potential environmental niche adaptation genes (Almeida et al. 2019). However, the genome size of mangrove endophytic *S. parvulus* VCCM 22513 was quite similar to its terrestrial counterparts including JCM 4068[T], 2297, and LP03 (~ 7.69 Mb compared to average of ~ 7.52 Mb). Moreover, the subsystems of VCCM 22513 were nearly identical with *S. parvulus* reference sequences in the functional categories. In addition, the mangrove endophytic VCCM 22513 shared with other strains a large core-genome that harbored all protein families encoding functions related to the basic biology and phenotypes of *S. parvulus* species. This was in contrast to actinobacterial strain *Kitasatospora* sp. SUK42 from *Antidesma neurocarpum* Miq that adapted to endophytic life-style through genomic reduction (Zin et al. 2021). Hence, it seems that environmental niche adaptation of *S. parvulus* may not be under selective pressures. As this is the first mangrove endophytic *S. parvulus* sequenced so far, more genome sequences of *S. parvulus* from different environmental niches are further required to confirm whether *S. parvulus* has open genome as a result of natural selection.

Genome mining demonstrated that *S. parvulus* VCCM 22513 effectively utilized both mycothiol and ergothioneine to keep the cytoplasm in a highly reducing state. Under oxidative stress conditions, LMW thiols form mixed disulfides with redox regulation and thiol-protection of proteins termed as *S*-thiolation, which plays an important role in protecting active site cysteine residues from irreversible oxidation (Tung et al. 2019). In parallel, oxidative stress is neutralized by the oxidation of LMW thiols that is later converted to their reduced states by specific enzymes (Tung et al. 2018). In soil-derived strains *S. coelicolor* A3(2) and *Nocardia asteroides*, one copy of *mshA*, *mshB*, *mshC*, and *mshD* resulted in the production of 3–6 μM mycothiol (Park et al. 2006; Newton et al. 2008). Interestingly, the marine actinomycete *Streptomycetaceae* CNQ530 was shown to produce approximately 50 μM mycothiol but its genome has not been sequenced yet (Newton et al. 2008). Using comparative

genomics, mangrove endophytic VCCM 22513 and *S. olivaceus* B-3009[T] were found to have a higher number of *msh* and *egt* genes than those of mangrove and terrestrial genomes. This inferred that maintenance of a highly reducing cytoplasm could be an important protective strategy of actinobacterial strains against the hostile environment of mangrove plants. Of note, some plants might also uptake extracellular ergothioneine as an additional antioxidant through symbiotic relationship with beneficial microbes (Samples and Balunas 2020). In support of the genomic findings, strain VCCM 22513 was notably more resistant to H_2O_2-induced oxidative stress as compared to endophytic *S. cavourensis* YBQ59. This led to a possibility that a highly reducing cytoplasm may be amongst the main factors that makes *S. parvulus* VCCM 22513 more resilient to oxidative stress triggered by *B. gymnorrhiza*. To our knowledge, this is the first genome mining report of *S. parvulus* describing biosynthetic pathways of mycothiol and ergothioneine.

Genes encoding ROS−scavenging antioxidant enzymes also contributed to oxidative stress resistance of strain VCCM 22513. Hydrogen peroxide-induced toxicity could be neutralized by VCCM 22513 due to the presence of catalases, thioredoxin system, superoxide dismutases, peroxidases, peroxiredoxins, and alkyl hydroperoxide reductases. All obtained genes are conserved across *Streptomyces* genera, indicating that enzymatic antioxidants are the driving force allowing *Streptomyces* to inhabit diverse environmental niches such as soils, lichens, and plants. The differences between VCCM 22513 and all compared genomes were the presence of additional copies of *ahp* and *bca* that catalyze the reduction of hydrogen peroxide and organic hydroperoxides to water (Jiang et al. 2019). Resistance to ROS in strain VCCM 22513 may likely correlate with single nucleotide polymorphism and other type of mutations that can improve positively the functioning of ROS-scavenging antioxidant enzymes, which is an interesting subject for future studies.

BGCs of desferrioxamin, coelichelin, melanin, and isorenieratene were present in the VCCM 22513 genome, further supporting the antioxidant properties of this strain. These compounds were previously found in the genome of *Streptomyces* sp. Babs14 and Osf17 isolated from the sand dunes of Sahara as an antioxidant strategy (Zerouki et al. 2021). Of note, desferrioxamin secreted by *S. parvulus* 03 from a mangrove plant is a specific iron-complexing agent completely preventing ROS production (Hu et al. 2018) which could contribute to the reducing power ability found in the VCCM 22513 extract. Also, a recent study proved that endophytic bacterial derived from mangrove plant *Avicennia marina* could protect tomato plant against the salinity-stress-induced oxidative stress via the production

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of peroxidases, superoxide dismutases, and catalases (Ali et al. 2017). However, the expression of BGCs under abiotic stress remains unknown. Future studies will be carried out to investigate the underlying mechanisms involved in non-enzymatic and enzymatic antioxidants of *S. parvulus* VCCM 22513 that help *B. gymnorrhiza* overcome oxidative stress under in vivo conditions.

An interesting outcome of *S. parvulus* VCCM 22513 genome analysis was the evidence of various genes participating in the catabolism of aromatic compounds. Since mangrove *B. gymnorrhiza* is a rich source of aromatic compounds due to its own production and pollutants uptake from the surrounding environments (Han et al. 2005; Naidoo and Naidoo 2016), *S. parvulus* VCCM 22513 must develop prorective mechanisms to resist and utilize these toxic substrates. As expected, strain VCCM 22513 grew well on the mineral medium supplemented with gentisate, cinnamate, styrene, and protocatechuate. It was in agreement with previous studies demonstrating that *Streptomyces* spp. were able to utilize benzaldehyde, catechol, phenylacetic acid, protocatechuic acid, and even polycyclic aromatic hydrocarbons as growth substrates (Khalil et al. 2019). Using genome-wide comparison, the complete pathways for degradation of aromatic compounds including protocatechuate, gentisate, 4-hydroxyphenylpyruvate, cinnamate, 3-phenylpropionate, and styrene were only identified in *S. parvulus* genomes including VCCM 22513 and JCM 4068[T]. In mangrove-derived *S. mangrovisoli* 149[T] and *S. olivaceus sp.* B-3009[T], the protocatechuate degradation pathway was predicted to be incomplete due to the absence of *catC*. Given that ROS could be generated by aromatic compounds (Alkio et al. 2005; Kim et al. 2007), the stress proteins of *Streptomyces scabies* 87.22 were highly upregulated in response to an aromatic compound such as ferulic acid (Khalil et al. 2019). In addition, the absence of mycothiol resulted in an inability to degrade gentisate and 3-hydroxybenzoate (Liu et al. 2013), indicating the importance of mycothiol as a cofactor of mycothiol-dependent maleylpyruvate isomerase NagL. Thus, the presence of genes encoding catabolic pathways along with antioxidants might render *S. parvulus* VCCM 22513 more adaptive to mangrove endophytic lifestyle.

Conclusion

Overall, the present study provided novel insights into adaptive mechanisms of mangrove endophytic VCCM 22513 to survive under oxidative hostile environment encountered while residing inside the host plant *B. gymnorrhiza.* Of the 15 endophytic actinomycetes, the isolate VCCM 22513 exhibited potent antioxidant activities against DPPH, superoxide radicals, and reducing power. Moreover, the strain was strongly resistant to H_2O_2, NaCl and able to grow in the presence of aromatic compounds. Based on the high-quality genomic information and comparative genome analysis, the endophytic strain VCCM 22513 was identified as a member of *S. parvulus* species. Genome-wide comparison revealed a highly conserved core genome of *S. parvulus* that might not be undergoing streamlining selection to adapt to environmental niches. Notably, *S. parvulus* VCCM 22513 harbored a wide range of genes encoding enzymatic, non-enzymatic, and secondary metabolites to overcome multiple stressors, including oxidative stress, high salinity, dramatic temperature changes, and toxic aromatic compounds. Thus, this work paves the way for a better understanding of underlying genetic mechanisms of *S. parvulus* adaptation to mangrove endophytic lifestyles, while enabling the discovery of potential antioxidant agents that can possibly be engineered for their future production.

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Availability of data and materials
Not applicable.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
The participant has consented to the submission of this article to the journal. We confirm that the manuscript, or part of it, has neither been published nor is currently under consideration for publication. This work and the manuscript were approved by all co-authors.

Competing interests
The authors declare that they have no competing interests.

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