Species Diversity of Cultivable Bacteria from Rhizophora stylosa Habitat and Their In Vitro Anti-Hepatitis B Virus Activity (Postprint)

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Abstract

Mangrove habitats harbor rich microbial flora, and their secondary metabolites exhibit novel structures, representing an important source for discovering new drugs. This study preliminarily evaluated the diversity of cultivable bacteria and their biological activities in Bruguiera gymnorhiza sediment, roots, leaves, and embryo axes, aiming to identify anti-hepatitis B virus (anti-HBV) drug-source strains. Pure culture techniques and 16S rRNA molecular biology techniques were employed to identify bacterial species and analyze species diversity. Using HepG2.2.15 cell line as a model, the anti-HBV activity of bacterial metabolites was tested via MTT and PCR techniques. LC-HRMS technology was utilized for preliminary analysis of metabolites from active strains. The results showed: (1) A total of 59 bacterial species were obtained in this study, belonging to 4 phyla, 5 classes, 14 orders, 23 families, and 36 genera, with Bacillus being the dominant genus. Strains GXIMD07402, GXIMD07665, and GXIMD07384 represent potential novel species of the genera Pseudooceanicola, Thioclava, and Aestuariibaculum, respectively. (2) Anti-HBV activity results demonstrated that extracts from GXIMD07366, GXIMD07616, GXIMD07384, GXIMD07550, and GXIMD07445X could significantly reduce HBV DNA levels in HepG2.2.15 cell supernatant ($P<0.05$), with inhibition rates of 51%, 47%, 63%, 52%, and 47%, respectively. (3) The highly active strain GXIMD07384's 4 major metabolites were preliminarily identified as Adenosine, Cyclo(L-Pro-L-OMet), Acremine G, and 7,8-dimethylbenzo[g]pteridine-2,4(1H,3H)-dione. These findings indicate that the cultivable bacterial community in Bruguiera gymnorhiza habitats exhibits rich species diversity and contains strains capable of producing anti-HBV active compounds, providing a foundation for the future application of marine microbial resources.

Full Text

Species Diversity and Anti-Hepatitis B Virus Activity of Culturable Bacteria from the Habitat of *Bruguiera gymnorrhiza*

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Abstract

Mangrove habitats harbor rich microbial flora whose secondary metabolites exhibit novel structures, representing an important source for discovering new drugs. This study preliminarily evaluated the culturable bacterial diversity in *Bruguiera gymnorrhiza* sediments, roots, leaves, and hypocotyls, and investigated the biological activity of bacterial metabolites to identify anti-HBV drug source strains. Pure culture techniques and 16S rRNA molecular biology were employed to determine bacterial species and analyze species diversity. Using the HepG2.2.15 cell line as a model, anti-HBV activity of bacterial metabolites was tested by MTT and PCR techniques. Secondary metabolites of active strains were preliminarily analyzed by LC-HRMS. The results showed: (1) A total of 59 bacterial species were obtained, belonging to 4 phyla, 5 classes, 14 orders, 23 families, and 36 genera, with *Bacillus* as the dominant genus. Strains GXIMD07402, GXIMD07665, and GXIMD07384 represent potential novel species of *Pseudooceanicola*, *Thioclava*, and *Aestuariibaculum*, respectively. (2) Anti-HBV activity assays revealed that extracts from GXIMD07366, GXIMD07616, GXIMD07384, GXIMD07550, and GXIMD07445X significantly reduced HBV DNA levels in HepG2.2.15 cell supernatants ($P<0.05$), with inhibition rates of 51%, 47%, 63%, 52%, and 47%, respectively. (3) Four major metabolites from the highly active strain GXIMD07384 were identified as Adenosine, Cyclo(L-Pro-L-OMet), Acremine G, and 7,8-dimethylbenzo[g]pteridine-2,4(1H,3H)-dione. These findings demonstrate that culturable bacteria in *B. gymnorrhiza* habitats exhibit rich species diversity and contain strains capable of producing anti-HBV compounds, providing a foundation for future application of marine microbial resources.

Keywords: *Bruguiera gymnorrhiza*, culturable bacteria, species diversity, metabolites, anti-HBV activity

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1. Introduction

Mangroves are unique coastal ecosystems characterized by intensive material and energy flow, high productivity, and vast microbial communities that serve as sources of new species and diverse bioactive compounds (Lin et al., 2020). As the largest group of microorganisms, bacteria hold significant value in industry and medicine. Jiang et al. (2018) isolated 101 endophytic actinomycetes from five mangrove species in Beilun Estuary, Guangxi, including seven potential novel species, with 31 strains showing positive antimicrobial activity and 21 exhibiting inhibitory effects against resistant pathogens. Li et al. (2021) isolated 71 culturable bacteria from the rhizosphere and tissues of *Cerbera manghas*, of which 15 strains displayed antifungal activity against agricultural pathogens, and all amplified at least one secondary metabolite biosynthetic gene from their genomic DNA. Li et al. (2018) obtained 32 bacterial strains from Hainan West Coast mangroves, including one new species and three strains with anti-aging activity against nematodes. These studies collectively demonstrate that mangrove bacteria represent a rich resource and important source of bioactive natural products worthy of further exploration.

Hepatitis B virus (HBV) is a hepatotropic DNA virus that causes chronic infection leading to cirrhosis and hepatocellular carcinoma, posing a serious threat to human health (Sarin, 2016). The global prevalence of HBsAg positivity reaches 3.9%, affecting approximately 292 million patients, with over one million deaths annually from end-stage diseases caused by HBV infection (Razavi-Shearer et al., 2018). In China, the HBV infection rate among the general population is 6.89%, with 83 million HBV patients and 300,000 deaths from chronic hepatitis B each year (Wang et al., 2019). Currently, the most effective treatments for hepatitis B are interferon and nucleoside analogs, but clinical applications suffer from significant side effects, high costs, drug resistance, and low cure rates (Zhang et al., 2015; Shi et al., 2017), making the search for novel and effective anti-HBV agents an urgent challenge in China. *Bruguiera gymnorrhiza* is a mangrove species in the family Rhizophoraceae with heat-clearing and detoxifying properties, and Jing nationality medical texts document its use for treating hepatitis B (Zhang et al., 2016). Our research group previously isolated seven cyanogenic glycosides from *B. gymnorrhiza* hypocotyls that inhibited HBV replication with IC_{50} values ranging from 5.1 $\pm$ 0.2 to 87.7 $\pm$ 5.8 $g \cdot mL^{-1}$ (Yi et al., 2015). However, the outstanding ecological value and poor renewability of mangrove resources constrain large-scale collection of *B. gymnorrhiza* hypocotyls for anti-HBV research. Microorganisms offer strategic advantages for drug development, including easy preservation, short growth cycles, and controllable metabolism, enabling large-scale fermentation. Currently,

few studies have reported culturable bacteria from *B. gymnorrhiza* habitats, and most have focused on antimicrobial activity. Ding et al. (2011) isolated four new ansamycin macrolides, divergolides A-D, from the endophytic *Streptomyces* sp. in *B. gymnorrhiza* stems, which showed strong inhibition against *Bacillus subtilis*, *Mycobacterium vaccae*, MRSA, and VRE. Yan et al. (2010) isolated *Streptomyces albidoflavus* from *B. gymnorrhiza* leaves and identified the first natural 8-acetoxy antimycin A18 with good fungicidal activity against plant pathogens, though anti-HBV activity was not reported. Therefore, this study investigates the species diversity of culturable bacteria from *B. gymnorrhiza* habitats and evaluates the anti-HBV activity of their metabolites to provide drug source strains for medicinal mangrove resource utilization and anti-HBV drug development.

2. Materials and Methods

2.1 Sample Collection and Preparation

In May 2019, *B. gymnorrhiza* habitat sediment, root, leaf, and hypocotyl samples were collected from the Beilun River Estuary Mangrove National Nature Reserve in Guangxi (108°14 11 E, 19°36 55 N). Sediment was excavated to a depth of 5 cm using a sterile shovel, while roots, leaves, and hypocotyls were directly collected and rinsed with sterile water to clean the surface. All samples were placed in sealed bags, stored in ice boxes, and transported to the laboratory for processing.

2.2 Culture Media

Ten isolation media were used: oatmeal medium (P3), tyrosine-asparagine medium (P7), trehalose-proline medium (M5), modified ISP5 medium (M7), arginine-asparagine medium (M9), modified starch-casein hydrolysate medium (M10), raffinose-histidine medium (M11), modified Gause's medium, R2A, and 2216E (AGG). Detailed formulations are referenced in Li et al. (2020). Purification medium consisted of modified ISP2 solid medium (yeast extract 2.0 g, malt extract 2.0 g, glucose 2.0 g, agar 20.0 g, and seawater 1,000 mL). Fermentation media included modified ISP2 liquid medium and modified ISP2 liquid medium supplemented with 0.1% *Chlorella*.

2.3 Cell Lines and Reagents

The HepG2.2.15 cell line was kindly provided by Professor Ye Li from the Guangxi Key Laboratory of AIDS Prevention and Treatment. Cell complete medium consisted of 90% DMEM, 10% fetal bovine serum, and 100 $U \cdot mL^{-1}$ penicillin-streptomycin. Chelex-100 resin and 2×Easy Taq Supermix were purchased from Bio-Rad (USA). 16S rRNA gene amplification primers 27F and 1492R were obtained from TransGen Biotech. Other reagents for isolation media were domestic analytical grade. DMEM medium, fetal bovine serum, penicillin-streptomycin, and biological grade dimethyl sulfoxide (DMSO) were

purchased from Gibco (USA). Thiazolyl blue tetrazolium bromide (MTT) was from Sigma (USA), lamivudine (3TC) from Shanghai Macklin Biochemical Technology, viral DNA genome extraction kits from Beijing Solarbio Science & Technology (China), and HBV DNA quantitative detection kits from Hunan Sansure Biotech.

2.4 Instruments

SW-CJ-2F clean bench (Suzhou Antai Air Technology), TAdvanced 96 PCR cycler (Biometra, Germany), ZWYP-2102 constant temperature shaker (Shanghai Zhicheng Analytical Instrument Manufacturing), HR1500- B2 biosafety cabinet (Qingdao Haier Biomedical), MCO-170AICDL-PC full-wavelength multifunctional microplate reader (Tecan, Switzerland), and LightCycler 480 II high-throughput real-time fluorescence quantitative PCR system (Roche, Switzerland).

2.5 Sample Pretreatment

Sample pretreatment followed the method of Li et al. (2018). *B. gymnorrhiza* roots, leaves, and hypocotyls were washed with sterile water, soaked in 75% ethanol for 2 min, and rinsed with sterile water to remove residual ethanol. Approximately 2.0 g of sediment and each plant tissue sample were ground in a sterile mortar with 2 mL seawater to prepare stock solutions, which were serially diluted with sterile seawater to 10^{-3} and 10^{-4} $g \cdot mL^{-1}$.

2.6 Bacterial Isolation and Purification

Aliquots (100 L) of 10^{-3} and 10^{-4} $g \cdot mL^{-1}$ dilutions were spread-plated on ten isolation media and incubated at 28 °C for 2–6 weeks. Distinct single colonies were picked and streak-purified on ISP2 medium using three-zone streaking. Well-grown, contamination-free strains were preserved in 20% (V/V) glycerol at 4 °C for short-term storage and -80 °C for long-term storage.

2.7 Molecular Identification

Genomic DNA was extracted using the Chelex-100 resin method (Zhou et al., 2010) based on Walsh et al. (1991) for PCR gradient amplification. Universal primers 27F and 1492R amplified 16S rRNA gene fragments. PCR products were detected by 1% agarose gel electrophoresis and sequenced by Shanghai Majorbio Bio-Pharm Technology (Guangzhou). Sequences were assembled using SeqMan software and analyzed via the EzBioCloud server (https://www.ezbiocloud.net/) (Kim et al., 2012) to obtain homologous type strain sequences. Venny online analysis was used to generate Venn diagrams of bacterial genera distribution from different *B. gymnorrhiza* sources at the genus level.

2.8 Metabolite Preparation and Screening

Log-phase strains were inoculated into two liquid fermentation media and cultured at 28 °C, 180 r · min^{-1} for 7 days. Fermentation broths were extracted with equal volumes of ethyl acetate, and extracts were concentrated under reduced pressure and stored at low temperature. HPLC-DAD analysis screened for metabolite-rich strains. ISP2 fermentation products were designated by bacterial code; ISP2 with 0.1% *Chlorella* products were designated "bacterial code X"; blank controls were named GXIMD00000 and GXIMD00000X.

2.9 Cell Proliferation Assay

Metabolite-rich strains were evaluated for anti-HBV activity. Extracts were dissolved in biological grade DMSO and diluted in complete medium to 500, 250, and 125 g · mL^{-1}. Log-phase HepG2.2.15 cells (5$×10^{4}$ cells · mL^{-1}) were seeded in 96-well plates (100 L/well), cultured for 24 h, then treated with test compounds. Blank and positive control (100 g · mL^{-1} 3TC) groups were included. After 72 h, supernatants were removed, 50 L of 5 mg · mL^{-1} MTT solution was added, and cells were incubated at 37 °C for 4 h. MTT solution was discarded, 100 L DMSO was added, and plates were shaken for 10 min. Absorbance at 490 nm was measured to calculate cell viability: Cell viability (%) = (A_{490} of experimental group / A_{490} of negative control) × 100%.

2.10 HBV DNA Detection

Strain extracts showing no cytotoxicity were selected for anti-HBV assays. HepG2.2.15 cells (5$×10^{5}$ cells · mL^{-1}) were seeded in 24-well plates (1 mL/well), cultured for 24 h, then treated with test compounds. On day 6, cell supernatants were collected, high-purity HBV DNA was extracted using viral DNA preparation kits, and HBV DNA levels were quantified using hepatitis B nucleic acid detection kits.

3. Results

3.1 Diversity of Culturable Bacteria from *B. gymnorrhiza*

Using ten isolation media, 59 culturable bacterial species were obtained based on colony morphology and 16S rRNA gene sequencing, with taxonomic distribution shown in . These 59 species belonged to 4 phyla, 5 classes, 14 orders, 23 families, and 36 genera. *Bacillus* was the dominant genus (9 strains, 15.3% of total isolates), followed by *Staphylococcus* (4 strains, 6.8%). Sequence alignment via EzBioCloud revealed three strains with low sequence similarity (<98.65%) to validly described species (Kim et al., 2014). Complete 16S rRNA gene sequencing (>1,330 bp) identified potential novel species: GXIMD07402 (GenBank MT613339) showed 96.33% similarity to *Pseudooceanicola aestuarii* E2-1 ; GXIMD07665 (GenBank MW709434) showed 96.24% similarity to both *Thioclava*

pacifica DSM 10166 and *Thioclava marina* MPZS01000005 ; and GXIMD07384 showed 98.52% similarity to *Aestuariibaculum suncheonense* SC17 .

3.2 Distribution of Bacteria in Different Samples

Bacterial distribution across samples is illustrated in [Figure 1: see original paper] and [Figure 2: see original paper]. Sediment yielded the richest diversity (27 genera, 40 species). Among plant tissues, roots contained the most species (12 genera, 17 species). Sediment was the primary source of Actinobacteria (10 genera, 14 species), with only *Brevibacterium casei* isolated from hypocotyls. Flavobacteria were exclusively isolated from sediment and roots. Except for *Pseudomonas stutzeri*, all γ-Proteobacteria (10 species) originated from sediment and roots. Hypocotyls and leaves showed lower bacterial richness, with 7 genera/13 species and 7 genera/9 species, respectively. Venn analysis at the genus level revealed *Bacillus* as the only genus common to all sample types. Sediment and roots shared seven genera (*Demequina*, *Myroides*, *Bacillus*, *Staphylococcus*, *Pseudooceanicola*, *Myroides*, *Vibrio*), showing greater overlap than other tissue combinations.

3.3 Isolation Efficiency of Different Media

The ten isolation media showed varying efficiencies ([Figure 3: see original paper]). Medium 2216E yielded the most strains (24) across the broadest genus diversity (11 genera including *Aestuariibaculum*, *Bacillus*, *Demequina*), and both novel strains GXIMD07402 and GXIMD07384 were isolated on this medium. Media M7 (20 species), R2A (16 species), and AGG (15 species) also showed high genus richness, all yielding *Bacillus*, *Microbulbifer*, *Pantoea*, *Staphylococcus*, and *Vibrio*. Media P3 and P7 yielded 14 species (9 genera) and 10 species (7 genera), respectively, with novel strain GXIMD07665 isolated on P7. The remaining four media (M5, M9, M10, M11) each isolated 5–8 species, all containing *Bacillus* and *Pantoea*. Based on strain number, genus richness, and species novelty, 2216E was the optimal isolation medium.

3.4 Effects on HepG2.2.15 Cell Proliferation

shows the effects of 25 metabolite-rich extracts and two blank media on HepG2.2.15 cell proliferation. At 125 $g \cdot mL^{-1}$, blank media showed no significant cytotoxicity compared to the control group ($P>0.05$). Among 25 fermentation products, 14 extracts exhibited no obvious toxic effects or proliferation inhibition, qualifying for subsequent HBV DNA suppression assays.

3.5 Inhibition of HBV DNA Secretion

Non-toxic extracts were evaluated for anti-HBV activity ([Figure 4: see original paper]). Compared to the blank control, both blank media showed no

significant reduction in supernatant HBV DNA levels (P>0.05). Strains GXIMD07366 (*Staphylococcus saprophyticus* subsp. *saprophyticus*), GXIMD07616 (*Pararhodobacter aggregans*), GXIMD07384 (*Aestuariibaculum suncheonense*), GXIMD07550 (*Demequina salsinemoris*), and GXIMD07445X (*Erythrobacter citreus*) significantly reduced HBV DNA levels (P<0.05) with inhibition rates of 51%, 47%, 63%, 52%, and 47%, respectively. GXIMD07384 showed the most potent anti-HBV effect, with HBV DNA reduction comparable to the positive control 3TC at 100 $g \cdot mL^{-1}$ (P<0.01).

3.6 Metabolite Analysis of GXIMD07384

GXIMD07384 (*Aestuariibaculum* sp.), a potential novel species, significantly inhibited HBV DNA replication in HepG2.2.15 cells. LC-HRMS analysis identified four major metabolites with retention times of 3.8, 11.7, 14.5, and 26.1 min and molecular ion peaks at $[M+H]^+$ 268.1047, 245.0957, 395.1819, and 243.0896, respectively. Based on secondary mass spectrometry fragmentation patterns, these compounds were tentatively identified as Adenosine (Kuchkarova et al., 2020), Cyclo(L-Pro-L-OMet) (Yang et al., 2013), Acremine G (Arnone et al., 2008), and 7,8-dimethylbenzo[g]pteridine-2,4(1H,3H)-dione (Yang, 2015) ([Figure 5: see original paper]).

4. Discussion and Conclusion

Mangroves harbor rich and unique microbial resources, and studying microbial species diversity is essential for utilizing mangrove and microbial resources. However, most marine microorganisms remain unculturable using current methods, partly because many exist in dormant states—a reversible, low-metabolic activity survival mode formed through long-term evolution (Mu et al., 2018). *Bacillus* species produce various bioactive enzymes including amylases, proteases, glucanases, cellulases, and chitinases, adapting them to the high organic matter environment of mangrove ecosystems and making them dominant in culturable mangrove bacteria (Sun & Lin, 2015; Zhao et al., 2018). This study isolated 59 strains from *B. gymnorrhiza* samples, belonging to 36 genera with *Bacillus* as the dominant genus. Despite substantial differences between laboratory and natural conditions that constrain traditional cultivation, we obtained diverse mangrove bacteria including three potential novel species belonging to *Pseudooceanicola*, *Thioclava*, and *Aestuariibaculum*. The *Aestuariibaculum* genus currently includes only three reported species (Jeong et al., 2013; Lee et al., 2013; Jiwon et al., 2018), all from marine habitats, thus expanding mangrove microbial resources. Advances in natural environment nutrient detection, metagenomic sequencing, and resuscitation mechanism studies may inform improved cultivation techniques to discover even richer microbial diversity.

Mangrove microorganisms exhibit antimicrobial, antiviral, antitumor, and antioxidant activities, representing an important source of bioactive natural products (Hong, 2013). Different mangrove species harbor distinct microbial communities that exchange information and genetic material with host plants through

long-term interactions, resulting in similar metabolic pathways and bioactive metabolites (Wang et al., 2014). Previous studies isolated anti-HBV compounds from *B. gymnorrhiza* plants. Using HBV-transfected HepG2.2.15 cells, we identified five bacterial strains that significantly reduced supernatant HBV DNA levels at 125 $g \cdot mL^{-1}$. These active strains belong to *Staphylococcus*, *Pararhodobacter*, *Aestuariibaculum*, *Demequina*, and *Erythrobacter*, with *Aestuariibaculum* showing remarkable anti-HBV activity. Both *B. gymnorrhiza* plants and their associated microorganisms produce anti-HBV substances. *Staphylococcus* strains carry the ISK-1 gene cluster encoding the immune protein NukH, which plays a synergistic role in host immunity (Sashihara et al., 2000). The *Aestuariibaculum* strain, as a rare and potentially novel genus with significant anti-HBV activity, warrants attention for producing potent antiviral metabolites during fermentation. LC-HRMS identified four major metabolites: Adenosine, Cyclo(L-Pro-L-OMet), Acremine G, and 7,8-dimethylbenzo[g]pteridine-2,4(1H,3H)-dione. Adenosine, a nucleoside compound, has demonstrated anti-HBV activity in vitro with an EC_{50} of 1.5 $mol \cdot L^{-1}$ (Dong & Chang, 2005). At 125 $g \cdot mL^{-1}$, the active strain achieved 63% inhibition, stronger than Adenosine alone, possibly due to synergistic effects among multiple active components. The active metabolites and mechanisms require further investigation, as do the ecological relationships between plants and microorganisms.

This study on culturable bacteria from *B. gymnorrhiza* habitats and their anti-HBV activity provides new medicinal resources and expands our understanding of microbial species diversity and pharmaceutical value associated with medicinal mangrove *B. gymnorrhiza*.

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Note: Figure translations are in progress. See original paper for figures.