Species Diversity of Actinomycetes from Rhizosphere Soils of Four True Mangrove Species on the West Coast of Hainan and Preliminary Screening for Anti-aging Activity Postprint

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Abstract

This study investigated the rhizosphere soils of four true mangrove species from the intertidal zone of the west coast of Hainan, analyzing the species diversity composition of mangrove rhizosphere actinomycetes and the bioactivity of their metabolic products, to accumulate a rich repository of actinomycete strains for the enhanced development and utilization of marine microbial resources. Nine different culture media were employed as isolation media, and strains were isolated and purified using pure culture methods combined with three-zone streaking, with diversity analysis performed based on actinomycete morphological characteristics and 16S rRNA gene sequencing results. The actinomycete fermentation broth was extracted with ethyl acetate, and its anti-aging activity was evaluated using Caenorhabditis elegans. A total of 22 actinomycete strains were isolated, affiliated with 4 orders, 7 families, and 9 genera, among which Streptomyces constituted the predominant group, and five strains (IMDGX 6012, IMDGX 6028, IMDGX 6118, IMDGX 6326, and IMDGX 6119) were preliminarily identified as potential novel species. The anti-aging activity assay of fermentation products revealed that metabolites from eight actinomycete strains significantly extended nematode lifespan ($P<0.05$); notably, IMDGX 6028 and IMDGX 6118, as potential new species belonging to Amycolatopsis and Curtobacterium respectively, exhibited extremely significant anti-aging activity ($P<0.01$), extending nematode lifespan by 22.2% and 26.6% compared to the control group. These findings demonstrate that the rhizosphere soils of true mangroves on the west coast of Hainan harbor abundant cultivable actinomycete resources and possess considerable potential for discovering novel actinomycete species and strains with anti-aging activity.

Full Text

Diversity and Anti-Aging Activity of Actinobacteria from True Mangrove Rhizosphere Soil on the West Coast of Hainan

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Abstract

This study investigated the rhizosphere soils of four true mangrove species from the intertidal zone of Hainan' s west coast to analyze the species diversity and metabolic activity of mangrove rhizosphere actinomycetes, aiming to accumulate abundant actinomycete strains for the exploitation and utilization of marine microbial resources. Nine different culture media were employed as isolation media, and pure culture methods combined with three-zone streaking were used to isolate and purify strains. Diversity analysis was conducted based on actinomycete morphological characteristics and 16S rRNA gene sequencing results. Fermentation broths were extracted with ethyl acetate, and anti-aging activity was evaluated using the *Caenorhabditis elegans* model. A total of 22 actinomycete strains were isolated, belonging to 4 orders, 7 families, and 9 genera, with *Streptomyces* being the dominant genus. Five strains—IMDGX 6012, IMDGX 6028, IMDGX 6118, IMDGX 6326, and IMDGX 6119—were preliminarily identified as potential new species. Anti-aging studies of fermentation products revealed that metabolites from 8 strains significantly extended nematode lifespan ($P<0.05$). Notably, IMDGX 6028 and IMDGX 6118, representing potential new species of *Amycolatopsis* and *Curtobacterium*, respectively, exhibited extremely significant anti-aging activity ($P<0.01$), extending *C. elegans* lifespan by 22.2% and 26.6% compared to the control group. These results demonstrate that the rhizosphere soil of true mangroves on Hainan' s west coast harbors rich culturable actinomycete resources with potential for discovering new species and anti-aging active strains.

Keywords: mangrove plants, rhizosphere actinobacteria, species diversity, anti-aging activity

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Introduction

Actinomycetes represent an important microbial resource for discovering novel secondary metabolites. Mangrove ecosystems, located in coastal intertidal zones, provide favorable conditions for microbial diversity due to their unique physicochemical environment and abundant humus (Sangkanu et al., 2017), and have been recognized as ideal habitats for isolating actinomycetes (Sweetline et al., 2012). Consequently, mangrove-derived actinomycetes have been a subject of intense research worldwide. Lee et al. (2014) isolated 87 actinomycete strains from tropical mangrove sediments in Malaysia, among which 5 were identified as new species. Hong et al. (2009) isolated 2,041 actinomycete strains from 112 soil and 99 plant samples collected across 8 mangrove sites in China, which were classified into 8 suborders, 11 families, and 25 genera, with some strains producing secondary metabolites exhibiting various pharmacological activities including antimicrobial, antitumor, and therapeutic effects against neurodegenerative diseases and diabetes.

China possesses rich mangrove resources, with Hainan Island being one of the major mangrove aggregation areas, hosting 38 mangrove species (Xin et al., 2016). The Dongzhaigang and Qinglangang mangrove reserves on Hainan' s east coast, as the largest mangrove distribution areas, have become hotspots for research, yielding multiple strains with antimicrobial, antitumor, and nematicidal activities (Li et al., 2016; Huang et al., 2013; Lei, 2006). In contrast, mangroves on Hainan' s west coast are primarily distributed in Lingao, Danzhou, and other areas, featuring smaller coverage and relatively simple community types compared to the east coast. Research on rhizosphere actinomycetes from this region remains scarce. Given the actual threats posed by human activities to mangrove resources, the timely high-value exploitation of rhizosphere actinomycetes from this area and the subsequent promotion of west coast mangrove ecosystem construction hold significant importance for China' s marine ecological civilization development. Furthermore, studies on the diversity of mangrove rhizosphere actinomycetes provide valuable references for evaluating the overall status of China' s marine microbial resources.

China' s population has shown negative growth trends in recent years, with aging becoming increasingly severe. It is projected that by the mid-21st century, the elderly population will reach 450 million, accounting for one-third of

the total population (Zeng et al., 2012), making the resolution of population aging issues urgent. Currently, most clinically used anti-aging drugs are synthetic compounds (Messing et al., 2013) with uncertain safety and efficacy. Mining new compounds from microorganisms has long been favored by scientists worldwide. Actinomycetes are considered among the most suitable microorganisms for discovering novel compounds, yielding new compounds including alkaloids, steroids, terpenoids, and polyketides, which have been reported for disease treatment and pest control (Singh et al., 2017). However, reports on health-promoting activities such as anti-aging effects for these identified compounds remain rare. This study analyzed the diversity of mangrove rhizosphere soil actinomycetes and preliminarily screened for strains exhibiting anti-aging phenotypes, providing important microbial resources for subsequent discovery of novel anti-aging compounds.

Materials and Methods

1.1.1 Mangrove Rhizosphere Soil Samples In July 2017, seven rhizosphere soil samples were collected from four true mangrove species on Hainan' s west coast. Sample details are provided in Table 1 . Sterilized shovels were used to excavate soil samples at a depth of 5 cm around each mangrove root system. Sampling locations for the same mangrove species were spaced more than 100 m apart. Soil samples were stored in ice boxes during transport and processed for strain isolation within 24 hours.

1.1.2 Nematode Samples *Escherichia coli* OP50 and wild-type *Caenorhabditis elegans* were kindly provided by Dr. Wang Bin from Guangxi Academy of Sciences.

1.1.3 Experimental Reagents Chelex-100 resin and 2×Easy Taq Supermix were purchased from BioRad (USA). 16S rRNA gene amplification primers 27F (5' -AGAGTTTGATCCTGGCTCAG-3') and 1492R (5' -GGTTACCTTGTTACGACTT-3') were obtained from TransGen Biotech (Beijing, China). 5% sodium hypochlorite was purchased from Langsuo Medical Disinfectant Co. (Hangzhou, China). All other reagents were domestically produced analytical grade chemicals.

1.2.1 Culture Media Formulations Isolation solid media: Following the method of Li et al. (2017), nine isolation media were employed: AGG, M4, M5, M7, M9, M10, M11, ISP7, and ISP3. Detailed formulations are available in the referenced literature.

Purification and preservation medium: 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 medium: Modified ISP2 liquid medium.

1.2.2 Processing of Mangrove Rhizosphere Soil Samples Following the method of Li et al. (2018), debris was removed from the soil surface, and 2.0 g of soil adhering to mangrove root surfaces was transferred to a flask containing quartz sand and 20 mL sterile water. The mixture was shaken thoroughly to prepare 10^{-2} and 10^{-3} dilutions. Aliquots (0.2 mL) of each dilution were spread onto nine isolation media and incubated at 28 °C for 2–8 weeks. Single colonies were selected and purified using the three-zone streaking method on ISP2 medium to obtain pure cultures, which were numbered and characterized for colony morphology and count. Purified strains were preserved in 20% (V/V) sterile glycerol at -80 °C.

1.2.3 Phylogenetic Analysis of 16S rRNA Gene Sequences Actinomycete genomic DNA was extracted using the Chelex-100 method (Zhou et al., 2010), and 16S rRNA gene sequences were amplified via gradient PCR (Walsh et al., 1991). Amplification quality and product size were evaluated by agarose gel electrophoresis. Target bands were excised and sequenced by Shanghai Majorbio Bio-Pharm Technology Co., Ltd. (Guangzhou Branch). Sequencing results were processed using DNA Star software, and the obtained 16S rRNA gene sequences were subjected to similarity searches and online alignment using the EzBioCloud database (https://www.ezbiocloud.net/) (Yoon et al., 2017) to identify the most similar validly published type strains as reference sequences.

1.3.1 Preparation of Crude Extracts from Mangrove Rhizosphere Actinomycetes Following the method of Qin et al. (2016), rhizosphere actinomycetes were activated on modified ISP2 solid medium and inoculated into four 200 mL flasks containing modified ISP2 liquid medium at their logarithmic growth phase. Fermentation was carried out at 28 °C with shaking at 180 rpm for 7 days. The fermentation broth was extracted three times with an equal volume of ethyl acetate, and the extracts were concentrated under reduced pressure and stored at low temperature in a desiccator.

1.3.2 Anti-Aging Activity Testing of Mangrove Rhizosphere Actinomycetes *Caenorhabditis elegans* was used as a model to screen the anti-aging activity of mangrove rhizosphere actinomycete fermentation products. Lifespan data were recorded (Lakowski & Hekimi, 1998) and analyzed using SPSS Statistics 17.0. Tables and graphs were prepared using Excel 2013 to evaluate the anti-aging bioactivity of actinomycete fermentation products.

Nematodes were washed with M9 buffer and centrifuged to remove the supernatant. Lysis solution (1 mL 5 M NaOH mixed with 0.5 mL 5% NaClO) was added to the nematodes at a 1:3 ratio, followed by shaking and centrifugation. Subsequently, 20 L *E. coli* fermentation broth, 30 L nematode pellet, and 150 L M9 buffer were added to each well of a 96-well plate, with negative con-

trols included. The plates were incubated at 20 °C for 48 h to obtain L4-stage nematodes.

Crude extracts were dissolved in 1% DMSO solution by sonication to a concentration of 500 $g \cdot mL^{-1}$. L4-stage nematodes were transferred to NGM medium (Brenner, 1974) containing 50 L of the test solution and incubated at 20 °C. Each group consisted of 2 plates with 20 L4-stage nematodes per plate, with day 0 defined as the start of treatment. Nematodes were counted every other day, and survival, death, and censored numbers were recorded. Nematodes were transferred to fresh plates until all had died.

Results

2.1 Diversity Analysis of Mangrove Rhizosphere Actinomycetes Based on colony morphology and 16S rRNA gene sequence analysis, 22 actinomycete strains were isolated and identified from 7 mangrove rhizosphere soil samples, representing 9 genera within 4 orders and 7 families. *Streptomyces* was the dominant genus, with 11 isolated strains. Detailed species information is presented in Table 2 . Strains IMDGX 6012, IMDGX 6028, IMDGX 6118, IMDGX 6326, and IMDGX 6119 showed highest sequence similarities of 97.75%, 98.15%, 98.32%, 98.44%, and 98.45% to the validly published type strains *Amycolatopsis lexingtonensis*, *Amycolatopsis niigatensis*, *Curtobacterium albidum*, *Curtobacterium citreum*, and *Demequina salsinemoris*, respectively. According to the taxonomic principle that strains with 16S rRNA gene sequence similarity below 98.65% represent potential new species (Kim et al., 2014), these five rhizosphere actinomycetes are likely novel taxa. IMDGX 6119 was isolated from *Avicennia marina* soil (2-2), while the other four potential new strains were all obtained from *Rhizophora stylosa* soil (1-14).

2.2 Distribution of 22 Actinomycete Strains Across Different Mangrove Soils and Media The distribution of 22 rhizobacterial strains across 7 different plant rhizosphere soil samples is shown in Figure 1 [Figure 1: see original paper]. Soil sample 1-14 yielded the highest number of isolates (10 strains) across 4 genera, including *Amycolatopsis*, *Curtobacterium*, *Sinomonas*, and *Streptomyces*, representing the most abundant and diverse collection. Sample 2-2 produced 4 strains belonging to 3 genera: *Demequina*, *Lysinimicrobium*, and *Streptomyces*. No actinomycetes were isolated from sample 2-1.

The isolation efficiency of seven culture media for mangrove rhizosphere actinomycetes is illustrated in Figure 2 [Figure 2: see original paper]. Results indicated that M11 and P3 media were most suitable for marine actinomycete isolation. M11 medium yielded 1 *Lysinimicrobium*, 2 *Amycolatopsis*, 5 *Streptomyces*, and 1 *Sinomonas* strain. P3 medium yielded 1 *Amycolatopsis*, 1 *Curtobacterium*, 1 *Microbacterium*, 1 *Demequina*, and 4 *Streptomyces* strains. Notably, *Amycolatopsis*, *Lysinimicrobium*, *Curtobacterium*, *Microbacterium*, and *Demequina*

are all rare actinomycete genera. Therefore, both M11 and P3 media demonstrated clear advantages for isolating mangrove rhizosphere actinomycetes in terms of both strain quantity and diversity. Additionally, *Amycolatopsis* grew on M5, P3, M9, and M11 media, while *Curtobacterium* was isolated on P7, P3, and M7 media.

2.3 Activity Analysis of Mangrove Rhizosphere Actinomycete Fermentation Products *Caenorhabditis elegans* is an excellent model organism for lifespan studies due to its clear genetic background, short lifecycle, minimal individual variation after synchronization, ease of culture and observation, and suitability for large-scale screening. Moreover, it shares 60–80% gene homology with humans (Park et al., 2017; Takuma, 2016). This study employed *C. elegans* to screen for anti-aging activity in crude extracts from 22 isolated actinomycete strains.

L4-stage nematodes were treated with actinomycete fermentation crude extracts to analyze their effects on survival lifespan (Table 3). Results showed that 8 of the 22 isolated strains exhibited anti-aging activity. Six strains—IMDGX 6017, IMDGX 6086, IMDGX 6014, IMDGX 6093, IMDGX 6013, and IMDGX 6182 —significantly delayed nematode aging, with *Streptomyces* showing the most prominent activity (5 active strains), suggesting its great potential for producing anti-aging substances. IMDGX 6028 and IMDGX 6118, isolated from *Rhizophora stylosa* soil as potential new species of *Amycolatopsis* and *Curtobacterium*, respectively, demonstrated the most significant lifespan extension effects, both prolonging L4-stage nematode lifespan by over 20%.

Discussion

With extensive exploitation of terrestrial resources, discovering novel microbial strains with unique biological activities has become increasingly difficult. Underexplored marine habitats represent rich sources of novel actinomycetes (Manivasagana et al., 2014). The unique mangrove ecosystem necessitates genetic-level adaptations for microorganisms to survive harsh growth conditions. One mechanism by which marine microbes adapt to extreme environments is activating silent genes to synthesize specialized bioactive secondary metabolites that maintain cellular physiological activity under high salinity, low temperature, and other stress conditions (Wilson & Brimble, 2009). Consequently, mangroves are also major producers of novel bioactive metabolites (Jiang et al., 2015). Since 2007, 66 new species and 8 new genera have been isolated and identified from mangrove environments, along with numerous new bioactive compounds produced by actinomycetes (Jiang et al., 2018).

The discovery of novel compounds has become increasingly challenging since the 20th century due to interference from numerous known compounds (Subramani & Aalbersberg, 2013). To obtain new active metabolites, investigating

microbial diversity in specific habitats is essential, with rare microorganisms deserving greater attention (Tiwari & Gupta, 2012). Subramani et al. (2013) similarly proposed that novel actinomycete taxa and rare actinomycetes should be prioritized for new compound discovery. Rare actinomycetes include not only naturally rare taxa but also difficult-to-culture strains (Stach, 2010). Therefore, selecting appropriate isolation media is critical. This study investigated actinomycete diversity in mangrove rhizosphere soils from Hainan' s west coast using different soil samples and culture conditions, isolating 22 strains, including 11 rare strains covering 8 genera. Five potential new species were identified, all belonging to rare actinomycetes: 2 *Amycolatopsis*, 2 *Curtobacterium*, and 1 *Demequina*. The genus *Amycolatopsis* reportedly contains over 20 secondary metabolic gene clusters that can be activated under different environmental conditions to synthesize specific secondary metabolites, conferring various biological activities such as immunosuppression and anticancer effects (Kumari et al., 2016; Peano et al., 2014). In this study, some actinomycete fermentation crude extracts significantly extended nematode lifespan, particularly the two novel rare strains, which prolonged lifespan by 20% at a crude metabolite concentration of 500 $g \cdot mL^{1}$. These findings further underscore the importance of novel strains, especially rare ones, in discovering new compounds. Previous literature has primarily focused on antimicrobial, anticancer, and macromolecule-inhibitory activities of actinomycete secondary metabolites, with rare reports on anti-aging activity. This suggests that these strains' metabolites may contain novel compounds responsible for their anti-aging effects. However, the specific active substances and mechanisms underlying the observed lifespan extension in *C. elegans* remain unclear and require further investigation, including compound isolation and identification, bioactivity analysis of pure compounds, and genomic studies of *C. elegans*.

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