Synthetic and Systems Biotechnology 10 (2025) 262–270

Contents lists available at ScienceDirect

Synthetic and Systems Biotechnology

journal homepage: www.keaipublishing.com/en/journals/synthetic-and-systems-biotechnology

Original Research Article

Harnessing the microbial interactions from *Apocynum venetum* phyllosphere for natural product discovery

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ARTICLE INFO

Keywords:
Natural products
Biosynthetic gene cluster
Co-culture
Metabolomics
Phyllosphere microbiota

ABSTRACT

Natural products (NPs) afforded by living-beings, especially by microscopic species, represent invaluable and indispensable reservoirs for drug leads in clinical practice. With the rapid advancement in sequencing technology and bioinformatics, the ever-increasing number of microbial biosynthetic gene clusters (BGCs) were decrypted, while a great deal of BGCs remain cryptic or inactive under standard laboratory culture conditions. Addressing this dilemma requires innovative tactics to awaken quiescence of BGCs by releasing the potential of microbial secondary metabolism for mining novel NPs. In this study, a universal strategy was proposed to induce the expression of silent BGCs by leveraging the dynamic interactions among coexisting microbial neighbors within a microbiota. This approach involves the deconstruction/reconstruction of binary interactions among the coexisting neighbors to create a pipeline for BGCs arousing. Coupled with the acquisition of 2760 microbial individuals from the *Apocynum venetum* (Luobuma, LBM) phyllosphere in a successive dilution procedure, 44 culturable isolates were screened using binary interaction, in which 12.6 % pairs demonstrated potent mutual interacting effects. Furthermore, after selecting the four most promising isolates, a full-scale metabolic inspection was conducted, in which 25.3 % of the interacting pairs showcased significant metabolomic variations with decryptic activities. Notably, with the aid of visualization of IMS technology, one of the physiologically functional entities, the bactericidal agent resistomycin, was elucidated from the core interacting pair between the co-culture of the *Streptomyces* sp. LBM_605 and the *Rhodococcus* sp. LBM_791. This study highlights the intrinsic interactions among coexisting microorganisms within a phyllosphere microbiota as novel avenues for exploring and harnessing NPs.

1. Introduction

Natural products (NPs) produced by microorganisms are pivotal for drug development, with their significance exemplified by the groundbreaking impact of the streptomycin in the treatment of clinical infectious diseases. Microbial natural products have gained ever-increasing attention, in which approximately 70 % of the clinically used antibiotics are currently derived from microorganisms, with two-thirds originated from actinomycetes especially [1]. This situation has reinforced the critical role of natural microbial products, particularly NPs from actinomycetes, in drug development. Nonetheless, traditional methods of NP discovery typically lead to frequent rediscovery of already-known chemicals, entwined by the emergence of antibiotic-resistance, highlighting the urgent desire for novel drug exploration [2].

To address this issue, two major approaches have been developed: expanding resources and innovating methodologies. Resource

Peer review under responsibility of KeAi Communications Co., Ltd.

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https://doi.org/10.1016/j.synbio.2024.11.002

Received 7 August 2024; Received in revised form 30 October 2024; Accepted 5 November 2024

Available online 8 November 2024

expansion involves exploring rare actinomycetes [3,4], extreme environmental species [5,6], and microbiota sources [7]. Among these, investigating NPs in microbiota, particularly in the phyllosphere microbiota of plants, has gained prominence. The phyllosphere is characterized by limited nutrients availability, unstable water supply, and scorching UV cosmic radiation, provides a specialized ecological niche where microorganisms engage in various interactions, including resource competition, pathogenic stress, community sensing, and horizontal gene transfer [8,9]. Numerous potent NPs have been identified in this unique biosphere. For example, by analyzing over 200 isolates in the phyllosphere microbiota of the *Arabidopsis thaliana*, researchers have identified the most active bacilli species. Among the isolated compounds were the anti-infectious and cytotoxic NPs, marthiapeptide A and streptocidin D, along with a rare lysophospholipid [10]. Furthermore, gramicidin S, known for its ability to inhibit plant pathogenic bacteria, was found in *Bacillus* isolates from the phyllosphere of Chinese cabbage [11]. Therefore, the phyllosphere microbiota is a valuable asset for discovering NPs.

In addition to expanding the reservoir of microbial resources, it is crucial to explore more efficient methodologies for microbial prospecting. Advances in DNA sequencing technology and bioinformatics have facilitated the discovery of tens of thousands of potential gene clusters involved in the biosynthesis of NPs. However, the fraction of these clusters successfully manipulated in laboratory settings remains limited [12]. Various strategies have been developed to overcome these limitations, categorized as culture-dependent or culture-independent [13]. Culture-independent approaches encompass the targeted engineering of organisms capable of producing the desired molecules through heterologous expression, homologous activation, and mutagenesis. For example, heterologous expression in *Streptomyces coelicolor* as a host has led to the production of active NPs like cypemycin, grisemycin, and actagardine [14]. Similarly, a method involving enhanced phosphorylation in the carrier protein modification steps has uncovered cryptic/silent microbial biosynthetic pathways, yielding novel five oviedomycin and halichomycin-like compounds from two strains [15]. Additionally, a new oxybenadienone, penicimutalidine, was generated by the mutagenesis of marine fungi using diethyl sulfate (DES) [16]. Conversely, culture-dependent strategies employ the one strain many compounds (OSMAC) method, epigenetic modification, and co-culture to broaden chemical diversity [17,18]. Of these, microbial co-culture has garnered significant attention because of its capacity to induce chemical novelty through interactions resulting in the production of previously unobserved NPs. Co-culturing *Tsukamurella* isolated from soil with *Streptomyces* resulted in the production of the antibacterial polycyclic polyketide compound, alchivemycin A, and the cytotoxic indolocarbazole alkaloid arcyriaflavin E [19].

Nevertheless, the conventional techniques used for the investigation and isolation of microbial NPs present challenges. These approaches cannot comprehensively examine the patterns of metabolite synthesis and release individual or mixed microbial samples. Furthermore, there is a growing demand for the rapid identification and acquisition of active molecules, particularly those with low yields that necessitate structural identification [20]. Compared to traditional TLC and HPLC technologies, the combination of MALDI-MS, LC-MS, and other technologies enhances detection sensitivity, facilitates chemical analysis in co-culture studies, and aids in the identification of activated molecules [21]. MS-based imaging methods have proven effective for analyzing metabolites from microbial interaction zones in co-cultures [22]. Sing-crystal X-ray diffraction technology has been increasingly used alongside traditional nuclear magnetic resonance spectroscopy for the structural characterization of NPs. Sing-crystal X-ray diffraction provides an intuitive visualization of compound structure and configuration, particularly beneficial for NPs with low yields and complex structures [23].

In this study, we combined resource expansion and methodology integration to explore a new platform for the discovery of NPs. *Apocynum venetum* (LBM) is a Chinese herbal folklore native to Xinjiang, with antioxidant, antiplatelet, antidiabetic, anticancer, hepatoprotective, and antibacterial properties. The leaves of LBM contain flavonoids, phenylpropanoids, polysaccharides, terpenes, organic acids, and coumarins, among other chemical compounds [24]. Therefore, we collected and analyzed the microbiota from the phyllosphere of the LBM. Through screening technologies such as LC-TOF/MS and MALDI-TOF/MS, microorganisms capable of activating a wide range of silent gene clusters in this microbiota were identified through co-cultivation. Subsequently structures of activated compounds were elucidated using large-scale fermentation and crystallographic technologies. Our findings demonstrate how co-culture can activate the expression of silent gene clusters and underscore the value of the phyllosphere microbiota in discovering active NPs.

2. Materials and methods

2.1. Collection of the LBM leaves

For this experiment, leaves of the LBM were collected from Alar, Xinjiang Uygur Autonomous Region (81.217548° E, 40.472687° N). The selected leaves were completely excavated and then sealed in sterile bags. They were transported to the laboratory under a temperature of 0 °C.

2.2. Extraction of total DNA from the leaves of the LBM

One gram of fresh LBM leaves from different parts was selected randomly and powdered into a fine powder using liquid nitrogen. The powder was then uniformly dispersed in 5 mL of sterile water. After removing plant tissues, the resulting filtrate was loaded into a pre-cooled EP tube and centrifuged at 13,800 *g* for 1 min to collect bacterial cells. Total DNA extraction was performed using a bacterial genomic DNA extraction kit (Shanghai Generay Biotech Co., Ltd) and stored at −80 °C. Metagenomic amplicon sequencing, using standard bacterial 16S rDNA V5~V7 region primers (**Table S1**), was conducted using the Illumina Miseq sequencing platform at the Personalbio Biotechnology Company.

2.3. 96-Well plate high throughput method for culturable isolates

To maximize the diversity of microbial species in the microbiota, including those with low abundance, we used a dilution procedure in 96-well plates to isolate cultivable strains in a laboratory setting. One-gram leaves were randomly selected from different parts. The leaf surfaces were sterilized three times for 30 s each in 1 % sodium hypochlorite and 75 % ethanol. Subsequently, the sterilized leaves were thoroughly grounded, where 10 mL of sterile water was added to evenly disperse the grinding fluid. The mixture was then shaked to fully release the endophytic bacteria. After allowing it to stand, the stock solution was diluted using a 10-fold gradient with 5 increments. The diluted solution was evenly spread onto solid plates containing Gause's Synthetic Agar, ISP4 (soluble starch 1.0 %, $(NH_4)_2SO_4$ 0.2 %, $CaCO_3$ 0.2 %, K_2HPO_4 0.1 %, $MgSO_4{\cdot}7H_2O$ 0.1 %, NaCl 0.1 %, and trace salt solution 0.1 % (v/v) containing $FeSO_4{\cdot}7H_2O$ 0.1 %, $MnCl_2{\cdot}4H_2O$ 0.1 %, $ZnSO_4{\cdot}7H_2O$ 0.1 %, agar 18 %, pH 7.2), ISP3 (oat flour 2 %, NaCl 0.1 %, and trace salt solution 0.1 % (v/v) containing $FeSO_4{\cdot}7H_2O$ 0.1 %, $MnCl_2{\cdot}4H_2O$ 0.1 %, $ZnSO_4{\cdot}7H_2O$ 0.1 %, agar 18 %, pH 7.2), and ISP2 (glucose 0.4 %, malt extract 1.0 %, and yeast extract 0.4 %, agar 18 %, pH 7.2) media. These plates were then cultured at 30 °C for 5 days. The number of microorganisms in each gram of LBM leaves was determined using the dilution counting method, and it was found to be 3.3×10^3. To ensure that the number of microorganisms in each well of the 96-well plate is equal to or less than one, 1 g of leaves was ground and diluted into 35 mL of sterile water to prepare a bacterial suspention for the 96-well plate. The 96-well plates were filled with 200 μL ISP4 solid medium to each well,

which contained nystatin at a concentration of 50 μg/mL. After the medium solidified, 10 μL of bacterial liquid was added to each well. The cells were then cultured at 30 °C for 5 days. The isolated strains were identified by 16S rDNA sequencing using primers 27F and 1492R (**Table S1**).

2.4. Binary interaction approaches

In this study, we cultivated 44 different strains from the microbiota of LBM leaves. These strains were grown on ISP4 medium at temperatures of 30 °C or 37 °C, which are the average high and low temperatures reported by the China Meteorological Administration at LBM collection sites (Fig. S1). To start the experiment, the frozen test strains were revived and incubated on solid medium. Then, they were used to inoculate liquid TSBY medium (tryptone soya broth 3 %, yeast extract 0.5 %, sucrose 10.3 %, pH 7.2) in shaking tubes. The turbidity of the seed was adjusted to an appropriate OD_{600} of 0.3–0.5. The strains were mixed with unsolidified ISP4 medium, and 1 μL of other strains was inoculated. After two to three days of incubation, we assessed the binary interactions. If the zone of inhibition, which is the distance between the outer edge of the halo and the colony's edge, exceeded 3 mm, we categorized the interaction as 'strong inhibition' (level 3). Smaller or partially cloudy halos were classified as level 2 or 1 inhibition. Additionally, we evaluated the growth of each strain. A promotion grade of 3 was assigned if the strain exhibited growth exceeding three times the size of the colony in separate cultures. Minor variations in strain growth were denoted by grades 2 and 1.

2.5. Fermentation of resistomycin in co-cultivation between LBM_791 and LBM_605

Strains were first activated, and individual colonies were selected. A seed culture with an optical density OD_{600} of 0.3–0.5 was prepared. Next, 500 μL of the dominant strain seed and co-culture strain seed were mixed thoroughly and evenly spread onto Petri dishes containing solid ISP4 medium. The dishes were then incubated in an inverted position at 30 °C and 37 °C for 5 days.

2.6. Sequencing of LBM_791 and LBM_605

The genomic DNA of the strain was extracted using a bacterial genomic DNA extraction kit (Shanghai Generay Biotech Co., Ltd) and sent to Personalbio (Shanghai Personal Biotechnology Co., Ltd.) for Nanopore Sequencing. The sequencing data were analyzed using the antiSMASH (Version 7.0, HYPERLINK "https://antismash.secondarymetabolites.org) online analysis software to determine putative natural product BGCs.

2.7. MALDI-TOF/MS for screening

The solid fermentation media were cut into small pieces and placed in a 50 mL tube. Methanol was added to extract the metabolites, followed by ultrasonication for 30 min. 0.5 μL sample and 0.5 μL HCCA matrix were then spotted on the polishing 384-well plate for MALDI-TOF/MS, in which HCCA solution acts as internal standard to calibrate the program. Followed this step, the instrument was calibrated with HCCA's multi-mass spectrometry molecular signal as a reference standard and adjusting the MALDI-TOF/MS laser signal within a range between 10^4 and 10^5 units. Finally, sample detection and analysis were conducted on different positions of each target hole using the FlexAnalysis software (Bruker).

2.8. Untargeted metabolomics screening via LC-TOF/MS analysis

The solid fermentation media were cut into small pieces and placed in a 50 mL tube. Methanol was added to extract metabolites, followed by ultrasonication for 30 min. The resulting mixture was filtered using a 0.22 μm organic filter and loaded into an injection vial. Metabolites in the fermentation broth were analyzed using HPLC-TOF/MS (Agilent 1290–6500 Q-TOF; Agilent, Santa Clara, CA, USA), with the following parameters: chromatographic column: Agilent TC-C18(2) (4.6 × 250 mm, 5 μm); detection wavelengths: 210 nm, 256 nm, and 365 nm; flow rate: 0.4 mL/min; injection volume:10 μL; detection mode: positive ion mode. Gradient elution was performed with ddH_2O (A) and acetonitrile (B) as mobile phases according to the following procedure: from min 0 to min 30, B ratio was increased from 0.5 % to 95 %; at min 33, the B ratio was set at 95 %; from min 33 to min 35, the B ratio was decreased from 95 % to 5 %. Data analysis was conducted using the Qualitative Navigator software.

2.9. Visualization of alternating molecular signals using mass spectrometry imaging technology

The target screening microbial seed solution and the TSBY medium containing 0.5 % agar were applied to a special electricity-conductive glass carrier for MALDI imaging. The solid medium had a thickness of 0.2 mm. After solidification, the sample was inoculated with the second interactive microorganism and incubated in a sterile environment for 24 h. Subsequently, the carrier plate was dried at 60 °C for 24 h. On the following day, the dried interaction plate was scanned before spraying a HCCA matrix onto the microbial interaction interface. The HTX TM-Sprayer (TMSP-M3) was used to evenly spray the matrix on the dried electricity-conductive carrier plate for imaging. The carrier plate was then completely dried and placed in a high vacuum state for MALDI analysis. Data were collected using a MALDI mass spectrometer and visualized and analyzed using the SCiLS lab software.

2.10. RNA extraction and transcriptional analysis

For transcriptional analysis, samples were collected at day 2, corresponding to the peak yield of resistomycin during the fermentation of LBM_605 and LBM_791 (Fig. S2). Mycelia were harvested from a 1 mL fermentation culture by centrifugation at 15,777 *g* for 10 min at 4 °C, then stored in liquid nitrogen for 0.5–1 h, and re-suspended in 1 mL of Redzol. High-speed homogenization (65 Hz, 30 s, repeated thrice) with glass beads was used to break down the mycelia. Subsequently, total RNA was extracted following manufacturer's instructions (SBS Genetech, Beijing, China). The quality and concentration of total RNA were analyzed using a NanoDrop 2000 spectrophotometer. DNase I (Thermo Fisher Scientific, Waltham, MA, USA) treatment was performed to remove contaminant DNA, and the resulting RNA was used as a template for PCR amplification using *remC*-F/*remC*-R primers (**Table S1**) to confirm the complete removal of DNA. Following confirmation, the RNA was used for cDNA synthesis using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Fisher), and the transcription of the core genes *remC* and *remN* (see Supplementary Information) in the resistomycin biosynthetic gene cluster was then analyzed using gel electrophoresis.

2.11. Dose-dependent correlation between co-culture species

Strain LBM_605 produces resistomycin. To investigate further, the addition amount of strain LBM_605 was kept consistent, while the addition of strain LBM_791 was varied. The inoculum was prepared following the aforementioned standards. Each of the four bottles of ISP4 medium inoculated with 1 mL of LBM_605, followed by the addition of 2 mL, 1 mL, 0.1 mL, or 0.01 mL of LBM_791 to each bottle. The mixture was then incubated at 30 °C and a speed of 220 r.p.m for three days. After incubation, 1 mL of the fermentation broth was extracted with 1 mL of methanol and sonicated for 30 min. The production of resistomycin was confirmed using LC-TOF/MS.

3. Results

3.1. Actinomycetes are prevalent within the LBM microbial community

To investigate the diversity of microorganisms within the phyllosphere of LBM, the total DNA of LBM leaf samples wase collected and extracted, in which the V5~V7 region of the 16S rDNA gene was amplified and sequenced using the Illumina MiSeq platform. In total, 93,176 bacterial sequencing reads were generated from all samples, which were filtered by removing low-quality sequences, chimeras, and singletons to obtain 77,034 high-quality reads. 16S rDNA amplicon sequencing data were classified into four phyla scattered into 42 bacterial amplicon sequence variants (ASV). The three most abundant phyla were Pseudomonadota (Proteobacteria), Actinomycetota (Actinobacteria), and Bacillota (Firmicutes), accounting for 75 %, 15 %, and 8 % of the total reads, respectively (Fig. 1A). The dominant genera identified in the samples at the genus level were *Halomonas* (72 %), *Nesterenkonia* (10 %), *Aliihoeflea* (4 %), and *Lactobacillus* (2 %) (Fig. 1B; Fig. S3). Culture-independent 16S rDNA amplicon sequencing revealed that the Pseudomonadota dominated the LBM phyllosphere microbiota at various taxonomic levels, comprising more than half of the overall abundance.

To maximize the diversity of microbial species in the microbiota, including those with low abundance, a dilution procedure in 96-well plates was conducted to isolate cultivable strains in a laboratory setting. Ultimately, we were able to isolate and purify 2760 individual microorganisms from 1 g of leaves. Following a preliminary morphological classification, full-length 16S rDNA sequencing was performed on 275 isolates and analyzed using the public database. Among the 275 sequenced microbial individuals, 44 single species were identified (**Table S2**), in which the Actinomycetota, Bacillota, and Pseudomonadota accounted for 43 %, 32 %, and 25 %, respectively. The most abundant strain was *Glycomyces* sp. LBM_636, followed by *Rosenbergiella* sp. LBM_610, *Streptomyces* sp. LBM_603, and *Pseudomonas* sp. LBM_620 (Fig. 1C). Actinomycetes were the predominant group among the isolated culturable strains, in contrast to the findings of the culture-free taxonomic sequencing. This discrepancy can be attributed to the selection of the culture medium and temperature. Given the robust secondary metabolism (SM) of actinomycetes and their significant role in producing microbial SMs, the enrichment of actinomycetes and co-culturable strains in the same environment was ideal to mine their SM potential.

3.2. Interacting pairs were screened among culturable isolates

Microorganisms originating from the same microbiota exhibit natural neighborhood coexistence. Previous studies have identified highly active interactions between microbial communities in *Arabidopsis* [10]. In this study, an experiment was conducted on the binary interactions among microorganisms isolated from the LBM phyllosphere, and analyzed through inhibitory or promoting activities.

To examine the network of interactions among the 44 bacterial isolates, a total of 1892 pairs of binary interactions were conducted, resulting in 238 pairs (12.6 % of all possible pairs) of interactions. These included 167 inhibiting pairs (70.2 %) and 71 promoting pairs (29.8 %) (Fig. 2; Figs. S3A and B). The *Bacillus* sp. LBM_47 displayed the most potent inhibiting effect with the index of 21, whereas the *Streptomyces* sp. LBM_605, *Acinetobacter* sp. LBM_841, and *Pseudomonas* sp. LBM_128 exhibited inhibiting effect with the index ranging from 10 to 20. The *Rhodococcus* sp. LBM_791 showed the most promoted properties, followed by the *Micromonospora* sp. LBM_562 and *Pseudomonas* sp. LBM_95. When considering both the promoted and inhibited performance index, strains with index exceeding 10 ranked from highest to lowest were LBM_47, LBM_605, LBM_841, LBM_791, LBM_128, LBM_562, LBM_874, and LBM_649. A high index indicates a strong survival advantage of the strain within the microbial community [10]. Statistical analysis was also conducted on the average genome size, GC content, type, and number of biosynthetic gene clusters at the genus level. The *Streptomyces*, *Micromonospora*, *Nocardia*, and *Rhodococcus* had the highest rankings and exhibited a strong potential for secondary metabolism (**Table S3**).

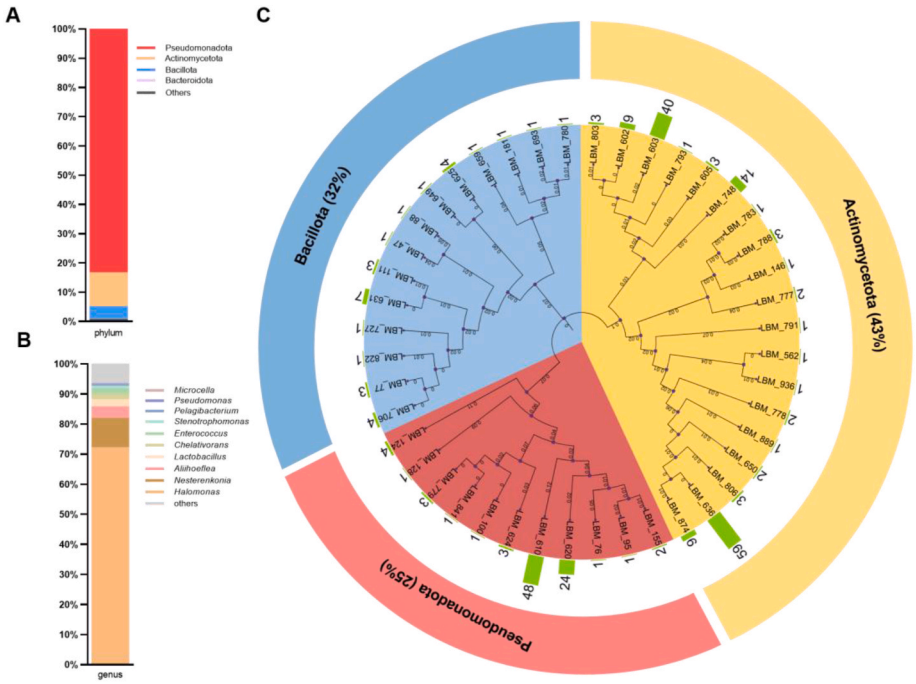

Fig. 1. Culture-independent 16S rDNA amplicon sequencing was used to classify the microbiota of LBM phyllosphere. A, diversity analysis of the top abundance phyla; B, genus-level diversity analysis of the top 10 abundances; C, diverse classification of culturable strains. The red refers to the Bacillota, yellow refers to the Actinomycetota, and blue refers to the Pseudomonadota. The phylogenetic tree and green histogram showed the isolated 44 strains and their abundance.

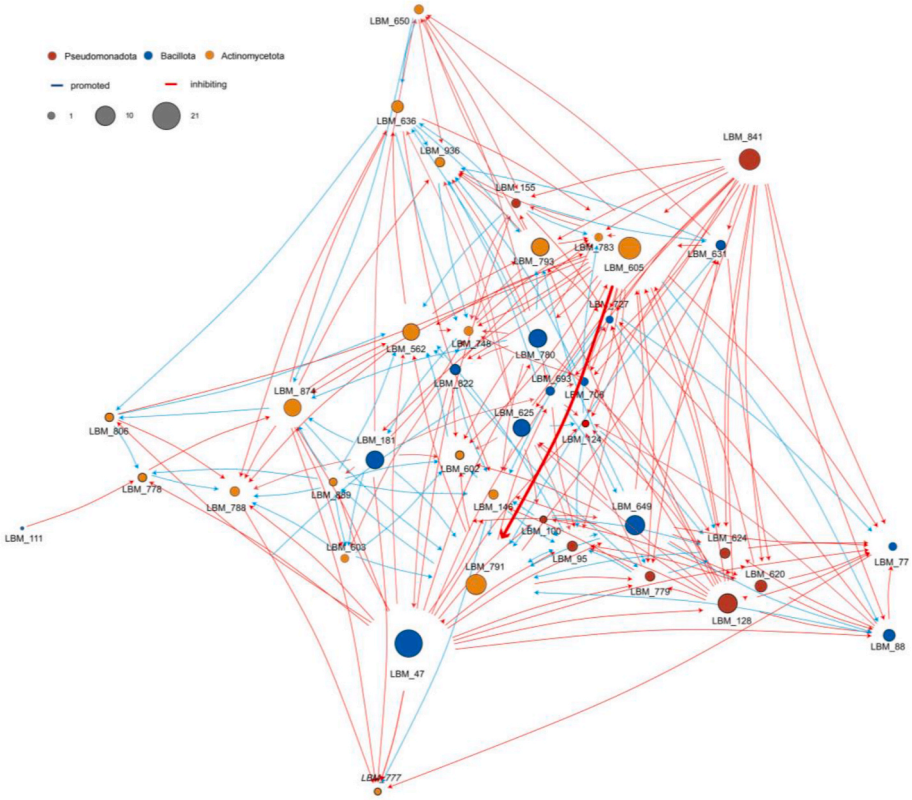

Fig. 2. Interactive screening of culturable microbial individuals from the LBM leaves. To construct the strain interactions within the LBM phyllosphere, the Davidson-Harel distribution algorithm was employed on the Hiplot platform. Each dot in the plot represents a specific strain, with red dots representing the Pseudomonadota, blue dots representing the Bacillota, and yellow dots indicating the Actinomycetota. The size of each dot corresponds to the number of interactions associated with that particular strain. Red lines connecting the dots signify combinations exhibiting inhibitory effects, while blue lines represent combinations displaying promoting effects. The direction of the arrow represents the interacting direction.

3.3. Core interacting pairs elicit universal metabolic shifts among coexisting neighbors

By combining the interaction effects and the metabolic potentials of these strains (**Tab. S4**), we identified four predominant actinomycetal strains in this group: *Streptomyces* sp. LBM_605, *Micromonospora* sp. LBM_562, *Rhodococcus* sp. LBM_791, and *Nocardia* sp. LBM_874. Based on the potential of co-culture to affect gene expression and its impact on strain interactions within the microbiota, a fine-tuned comparative metabolomic assessment was performed to characterize the interacting metabolic crosstalk between these four individuals and the remaining isolates by MALDI-TOF/MS, which provided a rapid and high-throughput detection of metabolic variations between mono-cultures and co-cultures. Taking the sampling circumstances of the Tarim Basin into consideration, the metabolic disparities between 332 sets of co-culture and mono-culture at 30 °C and 37 °C were characterized. Out of all the co-culture combinations, 84 pairs exhibited significant metabolic changes compared to the mono-cultures, accounting 25.3 % of the overall entries. This suggested that these four strains could induce notable metabolic changes when interacting with neighbors within the same microbiota (Fig. 3A; Fig. S4C; Tab. S4; Fig. S5). Analysis of the 84 pairs of co-culture combinations with observable metabolic alterations defined ten co-culture groups capable of activating secondary metabolites. The combination of LBM_791 and LBM_605 possessed the most productive capacity to diversely induce chemical entities (Tab. S5; Fig. 3B).

3.4. The activated molecule resistomycin was characterized by SCXRD in co-culturing of LBM_791 and LBM_605

Large-scale co-culture fermentation of LBM_605 and LBM_791 was further subjected to a preparative liquid chromatography and mass spectrometry to trace and purify the target activated compounds. The activated compound was characterized with the aid of crystallographic analysis, mass spectrometry and NMR, and proved to be a known natural product resistomycin, with a *m/z* of 377.1013 for $[M+H]^+$ in positive ion mode and a *m/z* of 375.0876 for $[M-H]^-$ in negative ion mode (Fig. 4A, B; Fig. S6–S8; Tab. S6–S7) [25–28]. According to the third-generation genome sequencing of LBM_605 and LBM_791, the biosynthetic gene cluster of resistomycin was assign to LBM_605, which revealed that the silent resistomycin biosynthetic gene cluster of the strain LBM_605 was induced by strain LBM_791. In addition, two methyltransferase genes (*remO* and *remP*) were identified in the resistomycin biosynthetic gene cluster of strain LBM_605 (Tab. S8), which leads the discovery of various methylated derivatives of resistomycin as confirmed by mass spectrometry analysis (Fig. S9) [29].

3.5. Physical contact serves as a major contributing-factor for activating effect

To explore the activating effect of the LBM_791 against the LBM_605 among co-culturing, MALDI mass spectrometry imaging (MALDI-MSI) was used to visualize the metabolic fingerprint of chemical crosstalk. As shown by MALDI-MSI, the activated resistomycin (*m/z* 377.1013) exhibited the strongest molecular signal at the interface where both the strains were in contact, gradually weakening as it moved away from the interface (Fig. 5A). To further investigate the eliciting effect of strain

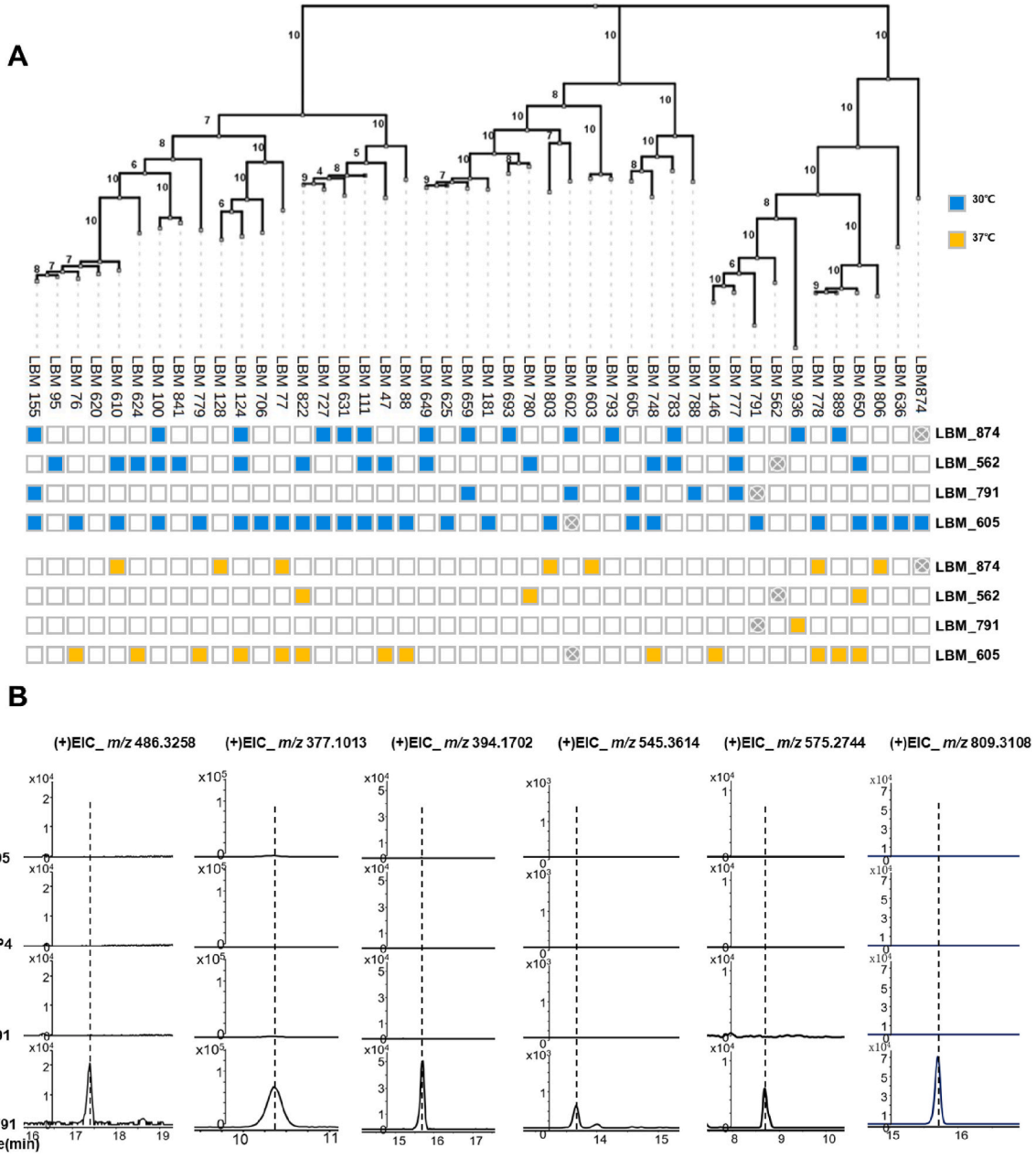

Fig. 3. The inspection of co-culturing broth under metabolomic content. A, the metabolic scenario of the co-culturing broth in the selected four isolates against the remaining strains as detected by MALDI-TOF/MS. Colors refer to metabolic shifts under different temperatures, cyan for 30 °C and orange for 37 °C. Construction of a maximum likelihood phylogenetic tree was performed using MEGA with 1000 bootstrap replicates; B, the metabolic comparison of EIC spectra in different molecules as detected by HPLC-TOF/MS. ISP4 stands for the medium, 605 for strain *Streptomyces* sp. LBM_605, 791 for strain *Rhodococcus* sp. LBM_791, and 605 + 791 for the co-culturing broth of *Streptomyces* sp. LBM_605 and *Rhodococcus* sp. LBM_791.

LBM_791, different amount of strain LBM_791 was introduced for co-culturing, while maintaining the quantity of LBM_605 constant. Based on the LC-TOF/MS detection, the yield of resistomycin was gradually accumulated in proportion to the increased biomass of inducing strain LBM_791 (Fig. 5B). Notably, transcriptional expression analysis of the resistomycin biosynthetic gene cluster in the LBM_605 demonstrated that the core genes *remC* and *remN* were up-regulated only in co-culture with the LBM_791 but not in monoculture (Fig. 5C and D). These observations inferred that the activation of the silent gene cluster in strain LBM_605 is completely depending on its coexisting with LBM_791, in a dose-dependent manner.

4. Discussion

Natural products have been successfully used as lead compounds for drug discovery [30]. From 1981 to 2019, the US Food and Drug Administration (FDA) approved 185 anticancer small molecules, of which 31.4 % were synthetic and 64.9 % were either NPs or directly derived from them [31]. In recent years, the discovery of natural products with new structures has decreased. Activating silent gene clusters in laboratory settings has become crucial in addressing this issue. Co-culture is recognized as an effective method for activating dormant gene clusters, but the random selection of traditional co-culture strains hinders the efficiency of this process. Additionally, challenges such as limited resources, low yields, and difficulties in extraction, purification, and structural elucidation pose obstacles in the field of natural

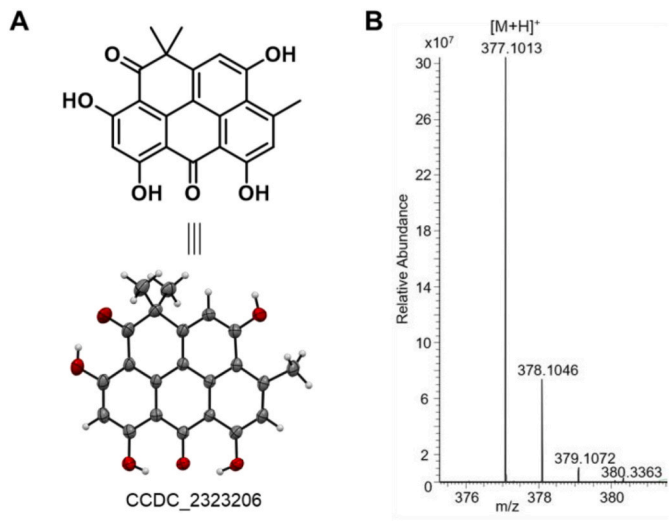

Fig. 4. Structural analysis of the resistomycin. A, the chemical structure and single crystal of the activated product resistomycin in an ORTEP drawing at a 50 % probability level. B, high-resolution mass spectrum of resistomycin in positive ion mode.

product mining [32].

In this study, we leveraged the phyllosphere of the LBM as a novel resource to explore NPs. Through co-culture, we successfully activated silent gene clusters and identified the resulting products using a combination of MALDI-MSI and liquid chromatography. Mass spectrometry imaging technology was employed to visualize small-molecule communication between microorganisms, alongside a tandem chromatography automatic separation system for the collection of the targeted compounds. Finally, we employed crystallographic techniques to elucidate the NP structures. This comprehensive methodology broadens the scope of microbial NP mining and introduces the concept of the microbiome in NP exploration.

Among the 332 co-culture combinations of LBM phyllosphere strains, 45 % exhibited significantly different metabolites compared to monocultures. This finding suggests that co-culture of strains from the same microbiota is an effective method for activating silent gene clusters. One of the identified co-culture-activated NP was resistomycin, an unusual aromatic polyketide metabolite with various pharmacologically properties, obtained from *Streptomyces* [33]. This finding suggests that resistomycin production may be linked to the competition for a niche within the microbiota, contributing to microbial community homeostasis [34].

In this study, *Rhodococcus* sp. LBM_791 was found to activate

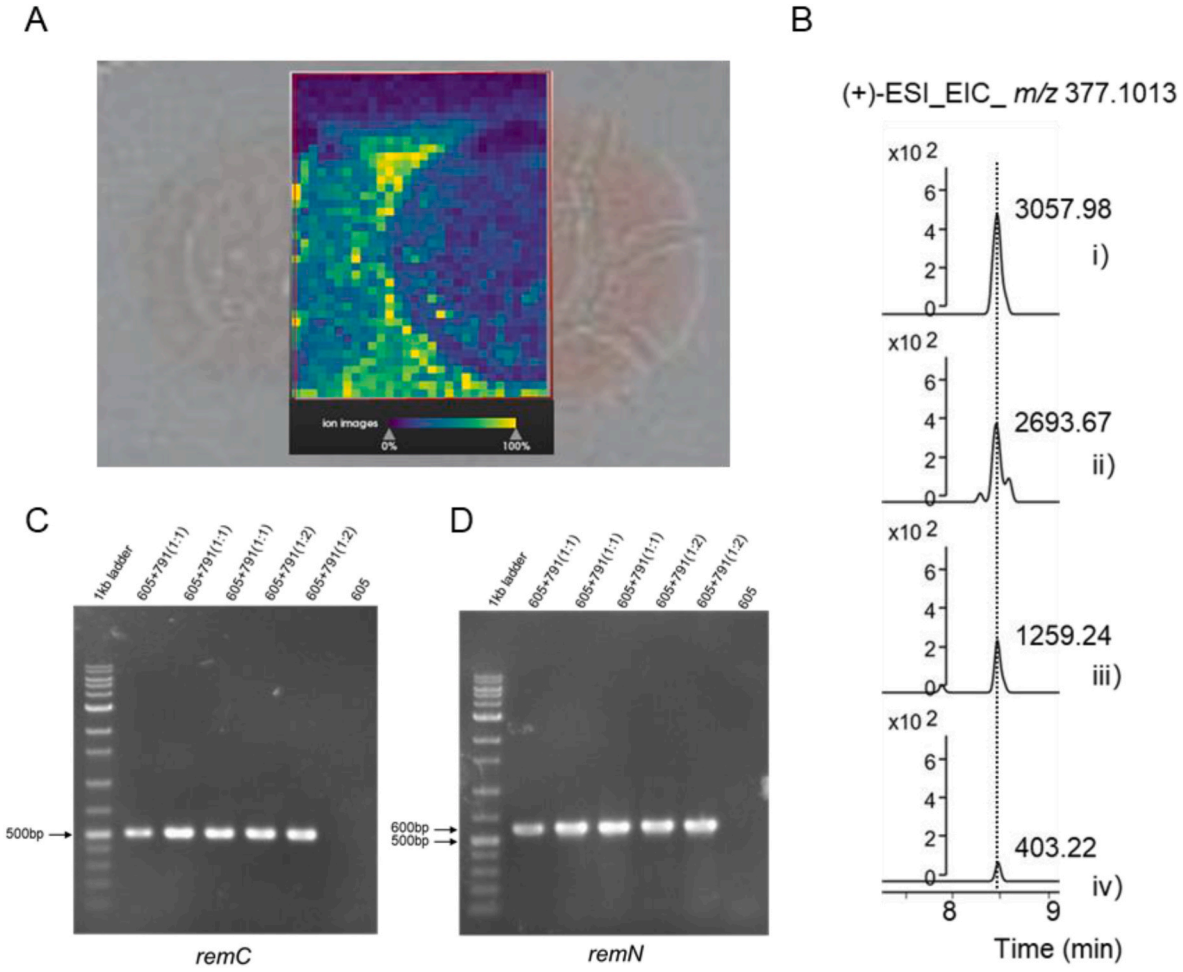

Fig. 5. The analysis of the activating effect for the strain LBM_791. A, visualization of resistomycin production during co-cultivation by mass spectrometry imaging; B, the production of resistomycin varied when different proportions of LBM_791 were co-cultured with the same amount of LBM_605. i) LBM_605 and LBM_791 in a 1:2 ratio. ii) LBM_605 and LBM_791 in a 1:1 ratio. iii) LBM_605 and LBM_791 in a 10:1 ratio. iv) LBM_605 and LBM_791 in a 100:1 ratio; C, the transcription of the *remC* gene in strain LBM_605 were evaluated by means of DNA gel electrophoresis, both in monoculture and co-cultured with strain LBM_791 at various ratios; D, the transcription of the *remN* gene in strain LBM_605 were evaluated by means of DNA gel electrophoresis, both in monoculture and co-cultured with strain LBM_791 at various ratios.

Streptomyces sp. LBM_605 to produce resistomycin. *Rhodococcus*, a genus of bacteria containing mycolic acid, affects the secondary metabolism of actinomycetes in laboratory culture [35]. However, it cannot be completely ruled out that other signaling molecules may also activate the silent gene clusters. Although the ecological processes driving this induction remain unclear, these results have significant implications for optimizing drug discovery, particularly for maximizing the extraction of SMs from existing microbial strain libraries [36]. In addition to resistomycin, we detected numerous molecules activated by co-culture using mass spectrometry, although their molecular structures remain unidentified. Nonetheless, this highlights the effectiveness of our activation strategy and underscores the potential of the leaves of LBM as a rich source of active molecules. The climate in Xinjiang exhibits significant temperature differences between day and night, along with strong ultraviolet light, providing an ideal environment for specialized natural products [37,38].

While we focused on bacteria-specific interactions, interactions between fungi-bacteria, fungi-fungi, and fungi-host can also activate complex chemical exchanges [39,40], warranting further exploration in future studies. Our metabolomic analysis demonstrated the reliability of the microbiota as a resource for mining NPs. Although our study observed only a small fraction of the vast array of NPs present in the LBM phyllosphere, several unidentified activating molecules require further investigation. Extensive research on the microbiota has revealed not only communication among microbial communities, but also intricate interactions and NP biosynthesis between the microbiota and its host organism, as well as its surrounding environment [41]. Integrating metabolomics, transcriptomics, proteomics, and other technologies holds promise for enhancing NP discovery within the microbiota, offering exciting avenues for future research in this field.

CRediT authorship contribution statement

Wei Huang: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Xingzhi Jiao:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Data curation. **Lingqi Hua:** Methodology, Investigation, Data curation. **Qianjin Kang:** Writing – original draft, Methodology, Investigation, Data curation. **Lili Zhang:** Writing – review & editing, Supervision. **Xiaoxia Luo:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Linquan Bai:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Data statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Funding

This work was financially supported by the Shanghai Municipal Science and Technology Major Project, National Key Research and Development Program of China (grant No. 2019YFA0905400), and National Natural Science Foundation of China (grant No. 31830104) to L.B.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The author Linquan Bai is an Editorial Board Member for *Synthetic and Systems Biotechnology* and was not involved in the editorial review or the decision to publish this article.

Acknowledgments

The authors thank Dr. Ningjin Zhang for single crystal XRD data collection and refinement at the Instrumental Analytical Centre of SJTU. They also thank the Instrumental Analysis Center of SJTU for providing services of compounds tracing and fermentation. They thank Dr. Wei Zhang in the Core Facility and Technical Service Center for School of Life Sciences and Biotechnology, SJTU, for help with LC-Q-TOFMS/MS data acquisition and data analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2024.11.002.

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