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BRIEF REPORT

ENVIRONMENTAL MICROBIOLOGY

Multi-omics analysis of antagonistic interactions among free-living *Pseudonocardia* from diverse ecosystems

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Abstract

Actinomycetes are a phylogenetically diverse bacterial group which are widely distributed across terrestrial and aquatic ecosystems. Within this order, the genus *Pseudonocardia* and their specialised metabolites have been the focus of previous ecological studies due to their antagonistic interactions with other microorganisms and their mutualistic interactions with insects. However, the chemical ecology of free-living *Pseudonocardia* remains understudied. This study applies a multi-omics approach to investigate the chemical ecology of free-living actinomycetes from the genus *Pseudonocardia*. In a comparative genomics analysis, it was observed that the biosynthetic gene cluster family distribution was influenced mainly by phylogenetic distance rather than the geographic or ecological origin of strains. This finding was also observed in the mass spectrometry-based metabolomic profiles of nine *Pseudonocardia* species isolated from marine sediments and two terrestrial species. Antagonist interactions between these 11 species were examined, and matrix-assisted laser desorption/ionisation-mass spectrometry imaging was used to examine in situ chemical interactions between the Southern Ocean strains and their phylogenetically close relatives. Overall, it was demonstrated that phylogeny was the main predictor of antagonistic interactions among free-living *Pseudonocardia*. Moreover, two features at *m*/*z* 441.15 and *m*/*z* 332.20 were identified as metabolites related to these interspecies interactions.

INTRODUCTION

In nature, actinomycetes exist in complex microbial communities, and the production of specialised metabolites underpins these interactions. In theory, it may therefore be possible to elicit specialised metabolite production in the laboratory, using co-culture experiments to mimic these naturally occurring interactions (van Bergeijk et al., 2020). For example, mycolic acid-containing bacteria have been shown to induce the production of antimicrobial metabolites in *Streptomyces* spp. (Hoshino et al., 2015; Onaka et al., 2011). Furthermore, interspecies interactions mediated by auto-regulators, such as γ-butyrolactones, explain the elicitation of metabolite production through actinomycete-actinomycete co-cultures (Kim et al., 2021; Niu et al., 2016). In general, the chemical interaction between actinomycetes in co-cultures involves complex regulatory mechanisms that influence the production of specialised metabolites (Kim et al., 2021; Traxler et al., 2013).

Ecological interactions are a strong force in the regulation of bioactive metabolite production. This species-specific antagonistic effect has been previously reported for the *Pseudonocardia* genus. For instance, *Pseudonocardia* strains in symbiosis with fungus-cultivating ants show stronger inhibition against the entomopathogenic fungus *Escovopsis* than against other fungi. Likewise, ant-related *Pseudonocardia* strains exhibited stronger *Escovopsis* inhibition than free-living *Pseudonocardia*, indicating their niche has

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driven specialised metabolism evolution (Cafaro et al., 2011), and mass spectrometry imaging (MSI) has shown that *Escovopsis* elicits the production of specific metabolites by ant-related *Pseudonocardia* strains (Gemperline et al., 2017). Similarly, *Pseudonocardia* also elicited the production of antimicrobial metabolites by *Escovopsis* during a response to a fungal infection (Heine et al., 2018). This complex specialised metabolite 'dance' makes these systems interesting for chemical characterisation and for the discovery of new bioactive metabolites.

Besides their antagonistic interactions against fungi, antagonistic interactions between *Pseudonocardia* strains in symbiosis with fungus-growing ants have also been described. Although there is evidence that fungus-growing ants contain a mixed actinomycetes consortium (Barke et al., 2010; Sen et al., 2009), the ants maintain and vertically transmit a single *Pseudonocardia* strain (Andersen et al., 2013; Poulsen et al., 2005). In this scenario, specialised metabolites active against other actinomycetes offer *Pseudonocardia* strains a competitive advantage to protect their ecological niche (Goldstein & Klassen, 2020). Recent reports suggest that vertically transmitted *Pseudonocardia* strains produce antibiotics that reduce the growth rate of other microorganisms, which selectively maintain an antibiotic-producing bacterial consortium (Worsley et al., 2021). Based on this ecological hypothesis, competitive assays between *Pseudonocardia* strains led to the discovery of the antimicrobial metabolites 9-methoxyrebeccamycin (Van Arnam et al., 2015) and the thiopeptide antibiotic GE37468 (Chang et al., 2020), both presumably related to a niche-defence function. However, the chemical ecology of marine-origin *Pseudonocardia* remains understudied, especially in the marine environment. In this study, metabolomics and genome mining tools were used to assess the role of the specialised metabolites on interactions between free-living *Pseudonocardia* isolated from marine environments.

EXPERIMENTAL PROCEDURES

Core-genome phylogeny

A total of 63 publicly available *Pseudonocardia* genomes in the National Centre for Biotechnology Information (NCBI) database were selected for the analysis (Table S1). Protein-coding regions and gene annotations were assigned using Prokka (v. 1.14.6) (Seemann, 2014). The orthologous genes shared across these genomes (*e*-value cut-off set to 1E−6) were then identified with Pangenome Iterative Refinement and Threshold Evaluation (PIRATE) (v.1.0.4) (Bayliss et al., 2019). The maximum-likelihood tree based on the core-genome (>95% genomes) alignment retrieved from PIRATE was reconstructed using FastTree (v.2.1.10) (Price et al., 2009) and visualised using iTOL (v.6.6) (Letunic & Bork, 2021).

Biosynthetic gene cluster annotation and analysis

Biosynthetic gene cluster (BGC) annotation was performed using antiSMASH (v.6.0) (Blin et al., 2021). Using the output, a sequence similarity network was generated by calculating pairwise distances between gene sequences and clustering them in gene cluster families (GCFs) using the Biosynthetic Gene Similarity Clustering and Prospecting Engine (BiG-SCAPE) (v.1.0.0) (Navarro-Muñoz et al., 2019) under hybrid and glocal alignment mode settings at a distance cut-off of 0.3. All available BGCs from MIBiG (v.2.0) (Kautsar et al., 2020) were integrated into the BiG-SCAPE analysis. The BiG-SCAPE output networks were visualised using Cytoscape (v.3.9.1) (Shannon et al., 2003).

Metabolite extraction and liquid chromatography–high-resolution mass spectrometry/mass spectrometry data acquisition

For each of the 11 *Pseudonocardia* strains (Table S2), an ISP2 broth pre-culture (100 μL) was inoculated on a Petri dish containing 20 mL of ISP2 agar supplemented with Instant Ocean® (18 g/L). After 28 days, samples were dried by lyophilisation (Labconco FreeZone 2.5 L Freeze Dry System) and extracted twice with 20 mL of ethyl acetate (Fisher Chemicals, UK) in 250 mL Erlenmeyer flasks with agitation (150 rpm) overnight. The extract was dried by Nitrogen flow, weighed and stored at 4°C. The dried extracts were reconstituted in acetonitrile Optima™ liquid chromatography–high-resolution mass spectrometry (LC/HRMS) (Fisher Scientific, UK) to a concentration of 1 mg/mL. Analysis using LC–MS using a Dionex UltiMate™ 3000 coupled to Q-Exactive™ (ThermoScientific, Germany) mass spectrometer with an electrospray ionisation source was carried out with a mass range of 70–1000 *m*/*z* in positive and negative ionisation modes with a spray voltage of 4.2 kV and capillary temperature of 310°C. A Phenomenex Kinetex 1.7 μm C18 100A (100 × 2.1 mm) column was used for chromatographic separation at 40°C. Mobile phase A consisted of H_2O with 0.1% formic acid (Fluka® Analytical, Switzerland), and mobile phase B consisted of acetonitrile Optima™ LC/MS (Fisher Scientific, UK) with 0.1% formic acid. A gradient was used, starting from 5% B and increasing to 50% from 0 to 8 min. Then to 99% from 8 to 10 min, and it was held constant for 2 min. Finally, B was brought back to initial conditions and held for 5 min for a total run time of 15 min. A flow rate of 300 μL/min and an injection volume of 10 μL were used.

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Classical molecular networking

A molecular network (Wang et al., 2016) was created using the online workflow (https://ccms-ucsd.github.io/GNPSDocumentation/networking/) on the Global Natural Product Social Molecular Networking (GNPS) website (http://gnps.ucsd.edu). The data was filtered by removing all MS/MS fragment ions within ±17 Da of the precursor *m*/*z*. MS/MS spectra were window-filtered by choosing only the top six fragment ions in the ±50 Da window throughout the spectrum. The precursor ion mass tolerance was set to 2.0 Da and an MS/MS fragment ion tolerance of 0.02 Da. A network was then created where edges were filtered to have a cosine score (normalised dot-product, which is a mathematical measure of spectral similarity between two fragmentation spectra. A cosine score of 1 represents identical spectra, while a cosine score of 0 denotes no similarity at all) above 0.7 and more than six matched peaks, according to the recommendations of Watrous et al. (2012). Further, edges between two nodes were kept in the network if and only if each of the nodes appeared in each other's respective top 10 most similar nodes. Finally, the maximum size of a molecular family was set to 100, and the lowest-scoring edges were removed from molecular families until the molecular family size was below this threshold. The spectra in the network were then searched against GNPS' spectral libraries. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a cosine score above 0.7 and at least six matched peaks. The molecular networks were visualised using Cytoscape software (v.3.9.1) (Shannon et al., 2003).

Intruder assay

The interactions between 11 *Pseudonocardia* strains from marine environments (Table S2) were studied in a challenge assay following the method proposed by Poulsen et al. (2007). Briefly, strains were pre-cultured in ISP2 broth (30°C, shaking at 150 rpm) for 14 days. First, the 'resident' strain was cultured in ISP2 agar supplemented with Instant Ocean® sea salt after adding 10 μL of the pre-culture in the centre of a Petri dish containing 20 mL of agar. After 28 days, the 'intruder' strain was inoculated around the resident strain colony from a 14-day liquid pre-culture using a sterile cotton bud. The zone of inhibition was measured after 14 days. The 11 *Pseudonocardia* strains were tested both as a resident and as an intruder strain in triplicate. Besides inhibition, morphological changes in colonies were also recorded. To visualise the results of the intruder assay in the context of the strains' phylogeny, multiple-alignment of almost complete 16S rRNA gene sequences was achieved using Clustal X (v.2.1) (Larkin et al., 2007), then a maximum-likelihood tree was built using Mega X (v.10.0.4) (Kumar et al., 2018) and visualised using iTOL (v.6.6) (Letunic & Bork, 2021).

MALDI-mass spectrometry imaging

Co-culture interactions were inoculated on ISP2 agar plates (8 mL in 9 cm Petri dishes) for 28 days at room temperature. The samples were excised and mounted on IntelliSlides conductive tin oxide glass slides (Bruker Daltonik GmbH). Each slide was pre-treated with 0.25 mL of 20 mg/mL 2,5-dihydroxybenzoic acid (DHB) matrix solution (two passes) using an HTX Imaging TM-Sprayer (HTX Technologies, USA), to prevent sample flaking (Yang et al., 2012). Images were subsequently taken using TissueScout (Bruker Daltonik GmbH) and a Braun FS120 scanner, followed by overnight drying in a desiccator. The following day, samples were covered with a matrix by spraying 2.5 mL of 20 mg/mL 2 DHB matrix solution (15 passes) in a Nitrogen atmosphere. The matrix solution was dissolved in acetonitrile/methanol/H_2O (70:25:5, v/v/v). Samples mounted on slides were dried in a desiccator overnight prior to measurement. Samples were analysed using a trapped ion mobility spectometer Time of Flight (timsTOF flex) (Bruker Daltonik GmbH) mass spectrometer in positive mode with a 100 μm raster width and a mass range of 100–2000 Da. Additionally, MS settings include: laser imaging 100 μm, power boost 3.0%, scan range 26 μm in the XY interval, and laser power 85%; tune: funnel 1 RF 300 Vpp, funnel 2 RF 300 Vpp, multipole RF 300 Vpp, in-source collosion induced dissociation 0 eV, deflection delta 70 V, Mattrix Assisted Laser Desorption/Ionisation (MALDI) plate offset 100 V, quadrupole ion energy 5 eV, quadrupole loss mass 100 *m*/*z*, collision energy 10 eV, focus preTOF transfer time 75 μs and pre-pulse storage 8 μs. Data were analysed using SCiLS software (v.2022b) (Bruker Daltonik GmbH).

RESULTS

Biosynthetic gene cluster analysis

As a model of free-living *Pseudonocardia*, the whole-genome sequence of three species (*Pseudonocardia abyssalis* KRD168, *Pseudonocardia oceani* KRD185 and *Pseudonocardia* sp. KRD291) isolated previously from the deep Southern Ocean (Millán-Aguiñaga et al., 2019; Parra et al., 2021) was selected and compared with *Pseudonocardia* strains from diverse ecological and geographical origins (Table S1). These strains correspond to two phylogenetically closely related species (*P. abyssalis* and *P. oceani*), and a third

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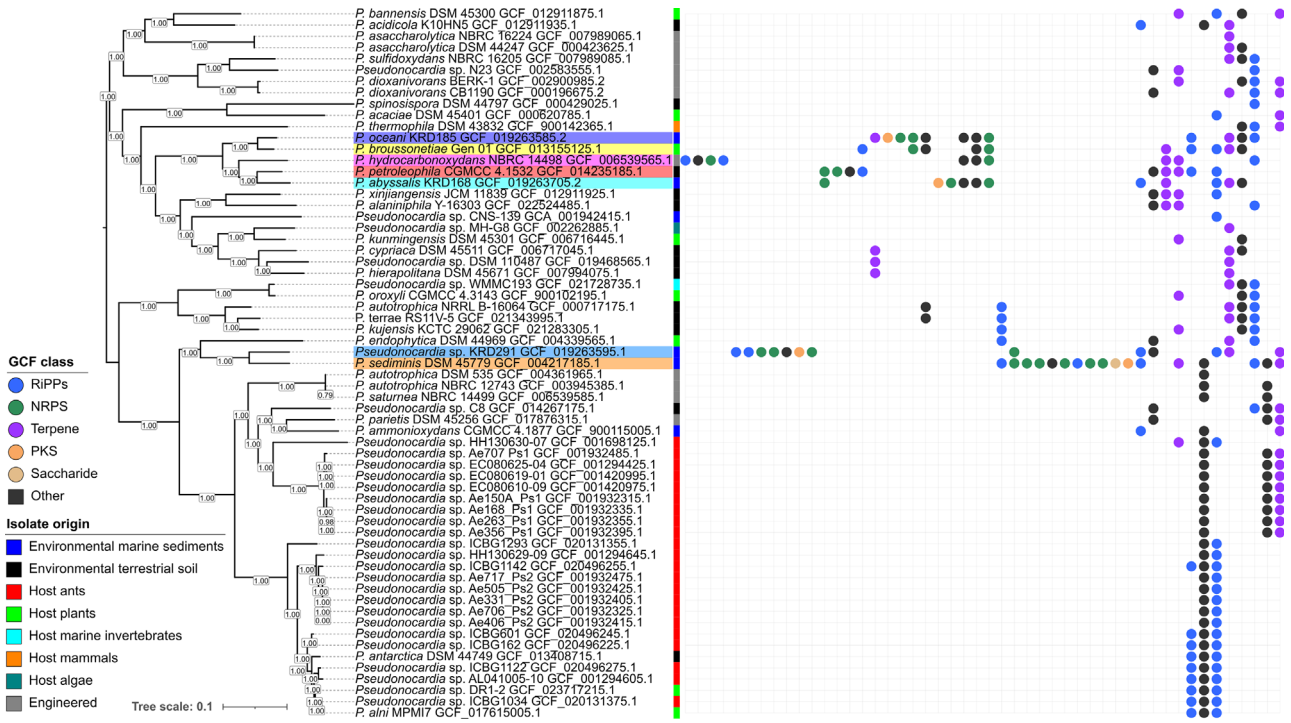

FIGURE 1 BGC distribution of selected *Pseudonocardia* strains grouped by core-genome (2 Mb) maximum-likelihood phylogeny. Nodes represent BGC according to natural product class. Node columns represent BGCs clustered in the same GCF according to the BiG-SCAPE analysis. The 'other' BiG-SCAPE class includes betalactone and ectoine. The Southern Ocean *Pseudonocardia* strains and their closest relatives are highlighted. The isolation environment of each strain is shown as a colour strip. Bootstrap support is indicated on the branches. Bar, 0.1 substitutions per nucleotide position.

species (*Pseudonocardia* KRD291), which is phylogenetically closely related to *Pseudonocardia sediminis*, another species of marine origin. The three Southern Ocean species were isolated from the same geographic area, representing an opportunity to study the geographical and evolutionary boundaries of the specialised metabolism within this genus. To better understand the evolutionary dynamics behind BGCs grouping into GCFs (Figure S1), the GCF absence/presence profile of the analysed strains was collated with their phylogeny reconstructed from a core genome (Figure S2). For the Southern Ocean species, in particular (Figure 1), the phylogenetic distribution of BGCs across the genus revealed a close correlation between the specialised metabolisms of *P. abyssalis* KRD168 and *P. oceani* KRD185, which have six GCFs in common. In general, evolutionary distance seems to have a greater influence on BGC distribution than the geographic or ecological origin of strains. For example, *P. oceani* KRD185 and *Pseudonocardia broussonetiae* Gen 01, an endophytic strain isolated from plant roots, shared 7 of the 12 GCFs, despite their different ecological origins. Similarly, *Pseudonocardia* sp. KRD291 only shared one GCF with *P. abyssalis* and *P. oceani*, despite their common geographic origin. Terpene, ribosomally synthesised and post-translationally modified peptides and ectoine BGCs showed a wide distribution across the genus, while non-ribosomal peptides, polyketides (PKs) and betalactone BGCs were common classes of species-restricted BGCs.

Intruder assay

To test the effect of the BGC distribution on interspecies interactions, antagonistic interactions between *P. abyssalis* KRD168, *P. oceani* KRD168, *Pseudonocardia hydrocarbonoxydans* DSM 43281 and *Pseudonocardia petroleophila* DSM 43193, as well as a further seven marine *Pseudonocardia* strains (Table S2) were evaluated (Figure 2). Strains were selected according to their phylogenetic relatedness, geographic origin diversity and culture collection availability. In general, it was found that antagonist interactions occurred more often between phylogenetically distant strains, which means that interactions between species of the same monophyletic group were not observed. For example, *Pseudonocardia antitumoralis* DSM 45322 was the most aggressive strain inhibiting 6 of the 10 challenger strains, (including *P. abyssalis* KRD168 and *P. oceani* KRD185), yet no antagonistic interactions against its phylogenetic neighbours (*Pseudonocardia ammonioxydans* DSM 44958 and *Pseudonocardia* sp. VO44-3) or the closely related clade of *P. sediminis* DSM 45779 and

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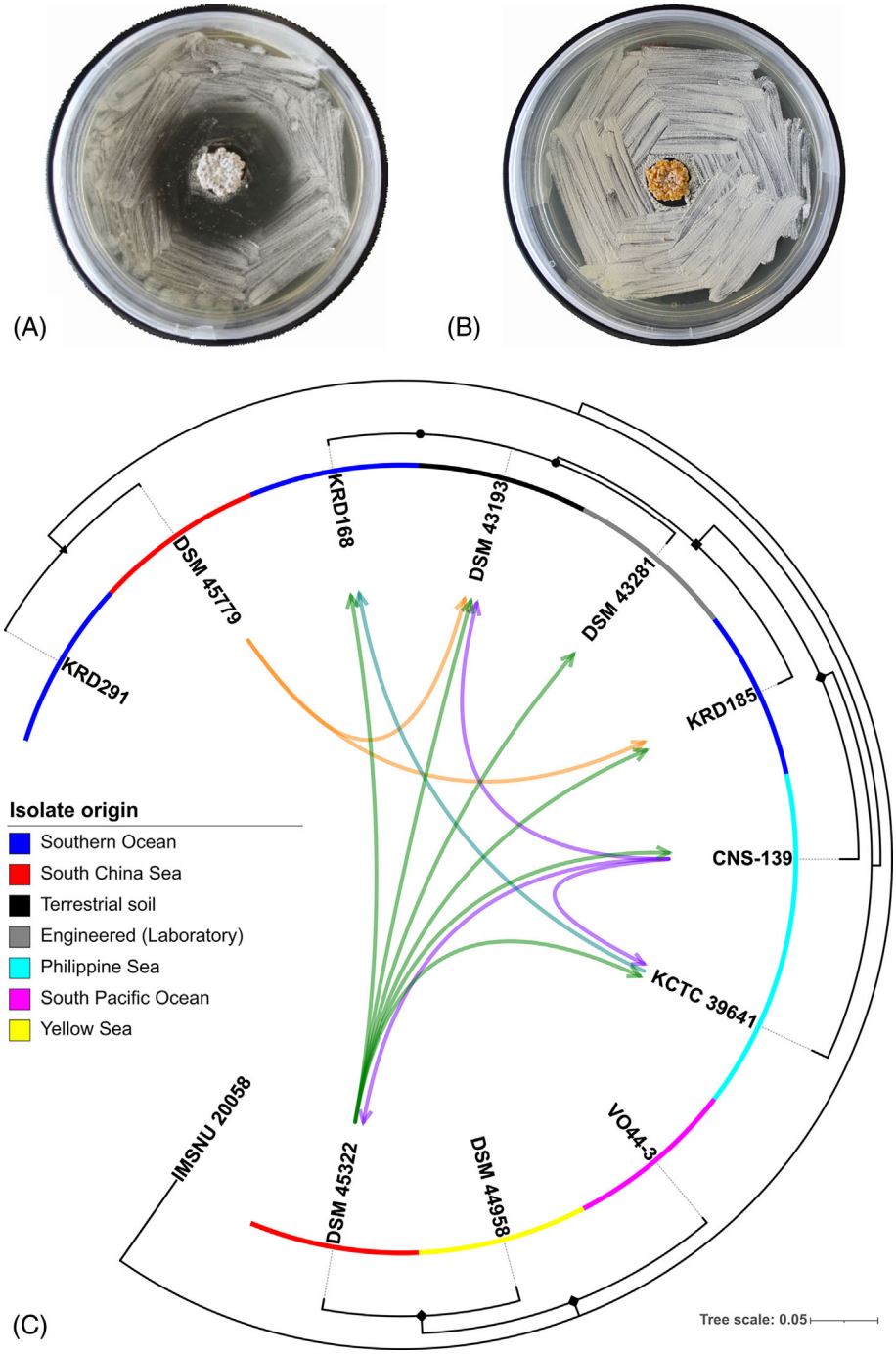

FIGURE 2 Intruder assay between free-living *Pseudonocardia* strains from diverse marine environments. (A) An example of an antagonistic interaction between *P. sediminis* cultured as a single colony (resident strain) and *Pseudonocardia petroleophila* inoculated 2 weeks later across the whole plate (intruder strain). (B) An example of the absence of antagonistic interaction between *Pseudonocardia oceani* (resident strain) and *Pseudonocardia abyssalis* (intruder strain). (C) Antagonist interactions mapped to *Pseudonocardia* phylogeny. A connecting line represents an antagonistic interaction of the resident strain (tail) against the intruder strain (arrow). Colours are used to distinguish intruder strains. Strains are distributed according to the maximum-likelihood tree based on the 16S rRNA gene sequence. *Amycolatopsis orientalis* IMSNU 20058 was used as an outgroup. Bootstrap support is indicated as symbols in the branches (▲ = 100, ■ >90, ♦ >80, ● >50). Bar, 0.05 substitutions per nucleotide position. The colour around the edge shows the strains' origin.

Pseudonocardia sp. KRD291 were observed. Similarly, *P. abyssalis* KRD168, *P. oceani* KRD185, *P. petroleophila* DSM 43193 and *P. hydrocarbonoxydans* DSM 43281, which are members of the same monophyletic group, did not interact with each other. It is important to point out that this experiment was designed to measure growth inhibition; additional morphological changes (e.g., sporulation or pigment production) because of

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interactions were not monitored. Although this approach focused on the production of antibiotics, the production of other specialised metabolites with functions such as communication, cannot be discounted.

Of the strains tested, phylogeny was the main factor in predicting antagonist interactions, however, our results also suggested the origin of the strains could also be involved. For example, none of the Southern Ocean strains antagonise each other, while *P. sediminis* DSM 45779 (originating from the South China Sea and phylogenetically close to KRD291) interacted against *P. oceani* KRD185. This may suggest that the Southern Ocean strains can share the same ecological niche despite their phylogenetic distance. Thus, ecological selection overcomes evolutionary distance when strains coexist.

Metabolomic analysis

A potential explanation for the intruder assay results is the metabolomic profiles of the interacting strains. From the classical molecular networking analysis (Figure S3), closely related strains shared a higher number of molecular features, indicating similar metabolites were detected. For example, *P. antitumoralis* DSM 45322 shared 1044 (72.0%) of its molecular features with *P. ammonioxydans* DSM 44958. Similarly, it shared 1045 (72.1%) molecular features with *Pseudonocardia* sp. VO44-3, and 1036 (71.5%) molecular features with *P. sediminis* DSM 45779 (Figure 3A) and 995 (68.7%) molecular features with *Pseudonocardia* KRD291 (Figure 3B). In two antagonistic interactions, *P. antitumoralis* shared only 429 (29.6%) and 391 (27.0%) parent ions with *P. abyssalis* KRD168 and *P. petroleophila* DSM 43193, respectively (Figure 3B).

Nonetheless, the metabolite distribution does not explain some of the observed antagonistic interactions. For example, *P. profundimaris* KTCT 39641 shared 815 (58.4%) molecular features with *P. abyssalis* KRD168 (Figure 3C), and an antagonistic interaction was observed. Similarly, *Pseudonocardia* sp. CNS-139 shared 539 (50.1%) molecular features with *P. profundimaris* KTCT 39641, and 552 (51.3%) with *P. petroleophila* DSM 43193 (Figure 3C). Still, *Pseudonocardia* sp. CNS-139 inhibited the growth of both strains. On the other hand, *Pseudonocardia* sp. CNS-139 shared a similar number of molecular features with *P. abyssalis* KRD168, 565 (52.5%), and no antagonistic interactions were observed (Figure 3C). These results showed that subtle differences in the metabolomic profiles could have significant consequences on bioactivity, as few metabolites could be responsible for the observed activity. In general, it was observed that metabolically similar strains (>70% similar) do not antagonise each other, while metabolically different strains

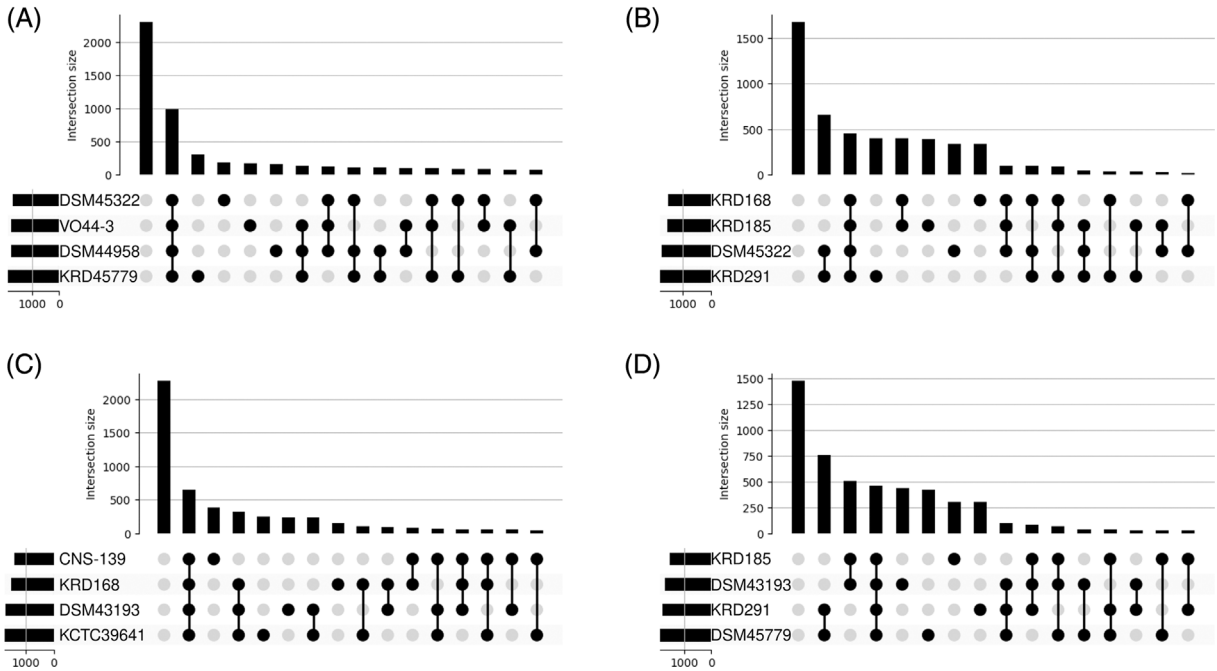

FIGURE 3 UpSet plot showing the node distribution from the GNPS classical molecular networking. Features with similar tandem mass spectrometry (MS/MS) spectra are clustered in the same node and can be interpreted as metabolites. The black dots at the bottom of each vertical bar indicate the intersection, which comprises strains that share the same nodes. The lined dots indicate that two or more strains share the same nodes. The vertical bars indicate the number of nodes of the corresponding intersection. The total number of nodes detected in each strain is represented by horizontal bars on the left. The total number of nodes in the molecular network is 5097 after applying the MS-Cluster algorithm to 98,935 spectra.

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(<30% similar) do. Otherwise, antagonistic interactions between metabolically similar strains (around 50% similar) cannot be directly predicted from the metabolite profile of monocultures.

A similar discrepancy regarding metabolically similar strains was observed in the case of *P. oceani* KRD185, *Pseudonocardia* sp. KRD291 and *P. sediminis* DSM 45779 as mentioned above. Interestingly, *Pseudonocardia* sp. KRD291 shared 363 (24.1%) parent ions with *P. oceani* KRD185 nodes, and no antagonistic interaction was observed, while *P. sediminis* DSM 45779 shared 359 (22.2%) with *P. oceani* KRD185 and showed an antagonistic interaction (Figure 3D). This means that *P. oceani* KRD185 and *Pseudonocardia* sp. KRD291 are metabolically different but can coexist. Therefore, as it was suggested for phylogeny distance, the metabolite profile is an important factor in explaining antagonistic interactions only in strains of an ecologically different origin, supporting the hypothesis that the ecological niche plays a significant role in the antagonism among free-living *Pseudonocardia* of marine origin.

Mass spectrometry imaging

As mentioned above, the metabolomic profiles of monocultures cannot fully predict antagonistic interactions in co-cultures. This situation could be expected as the metabolomic profile does not distinguish between basal and specialised metabolism. For instance, it is not possible to state that the metabolites produced in the monocultures and observed in the LC–MS/MS data are also present in the Petri dish during the intruder assay, which represents the major limitation of this analysis. To address this, MSI was used to detect the in situ production of metabolites during co-cultures. Using MALDI-MSI, the metabolite production of 28-day old co-cultures of *P. abyssalis* KRD168, *P. oceani* KRD185, *P. petroleophila* DSM 43193, *P. hydrocarbonoxydans* DSM 43281, *P. sediminis* DSM 45779 and *Pseudonocardia* sp. KRD291 was analysed. Through this approach, a metabolite of *m*/*z* 441.15 produced by *P. abyssalis* KRD168 and *P. oceani* KRD185 was identified in co-cultures and monocultures (Figure 4). Based on the BGC analysis, it is possible to

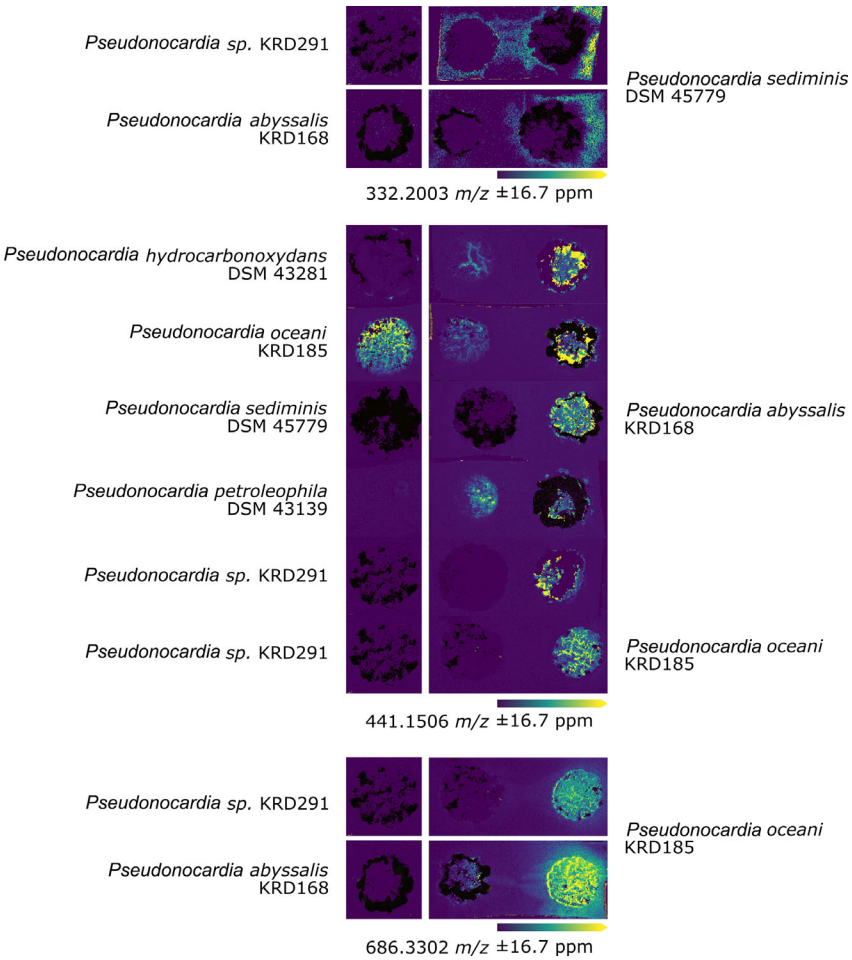

FIGURE 4 MALDI-TOF mass spectrometry imaging spatial detection of selected metabolites (*m*/*z* 332.20, 441.15 and 686.33) produced by *Pseudonocardia* strains during pairwise interactions. Each ion signal is normalised by the total ion count, and the colour scale represents the relative intensity for each signal. Mass-to-charge ratio (*m*/*z*) values are shown in the first column.

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propose that this metabolite may be related to the GCFs shared exclusively by both Southern Ocean species. Unfortunately, this metabolite was not observed in the LC–MS data and its identity remains to be confirmed. Another metabolite of *m*/*z* 748.58 was also detected mainly in the *P. abyssalis* KRD168 and *P. oceani* KRD185 cultures, but in this case, the intensity differences with other cultures were less clear (Figures S4 and S5). Similarly, a metabolite of *m*/*z* 686.33 was observed to be produced exclusively by *P. oceani* KRD185 (Figure 4), suggesting a specialised function. This metabolite may have some relationship with the NPRS and PKS BGCs annotated only in *P. oceani* KRD185 during the genomic analysis. However, the intensity of the production of this metabolite showed no differences between mono- and co-cultures (Figure S5), suggesting no induction by competition. Furthermore, a metabolite of *m*/*z* 332.20 that matches NPA012880—CJ-13,217 in the NPAtlas database was visualised in the *P. sediminis* DSM 45779 co-cultures and monoculture (Figure 4). This metabolite was not observed in the *Pseudonocardia* sp. KRD291 monoculture but seems to be induced by *P. sediminis* DSM 45779 during the co-culture. This potential auto-induction that is required by *Pseudonocardia* sp. KRD291 might explain differences in the results of the challenge assays despite the phylogenetic relatedness between these two species. Other metabolites observed in the MSI analysis include *m*/*z* 840.42 and *m*/*z* 1008.49, which were observed only in the *P. abyssalis* KRD168 cultures, *m*/*z* 257.14 was observed with higher intensity in *P. sediminis* DSM 45779 and *Pseudonocardia* sp. KRD291 cultures and *m*/*z* 639.31 produced only by *P. hydrocarbonoxydans* DSM 43281 (Figure S4). Box and whisker plots are present in the Supporting Information (Figures S5 and S6) for the metabolites described in Figures 4 and S4.

DISCUSSION

Despite all the available information about the chemical ecology of *Pseudonocardia* related to fungus-growing ants (Batey et al., 2020; Menegatti et al., 2021; Molloy & Hertweck, 2017; van der Meij et al., 2017), little is known about the interactions between free-living *Pseudonocardia*. Previously, Poulsen et al. (2007) proved that the antagonisms between strains of *Pseudonocardia* related to fungus-growing ants are common (Poulsen et al., 2007). In the current study, the same antagonism setup was replicated using 11 free-living *Pseudonocardia* strains, of which nine were isolated from marine environments. Poulsen et al. (2007) and Poulsen and Currie (2010) reported several antagonistic interactions between *Pseudonocardia* strains, including between the free-living strains *Pseudonocardia saturnae*, *Pseudonocardia halophobica* and *Pseudonocardia thermophilia*. In contrast, few interactions were observed between the marine-origin *Pseudonocardia* strains, most of these interactions involved *P. antitumoralis* DSM 45322. However, it was also reported by Poulsen et al. that the relative genetic distance between pairs of strains had a significant effect on antagonistic interactions. So, in general, the ecological and biogeographical variables had a small effect on describing antagonistic interactions.

Although phylogeny was more important than geographic origin to explain the observed antagonistic interactions, *P. sediminis* DSM 45779 and *Pseudonocardia* sp. KRD291 observationally showed different bioactivity profiles, which implies that the strain origin may play a role in the interaction against other *Pseudonocardia* strains. None of the three Southern Ocean species showed antagonistic interaction with each other, which suggests that for this group of actinomycetes, the geographical origin could have a role in the delimitation of the ecological niche. This observation was later confirmed by MSI, where *P. sediminis* DSM 45779 affected the production of certain metabolites in *P. abyssalis* KRD168 in a different way than *Pseudonocardia* sp. KRD291. These findings contrast with the observations made in soil-derived *Streptomyces* where antagonistic interactions occurred more often between co-occurring microbial populations (Kinkel et al., 2014).

As closely related species are more likely to have overlapping metabolic coverage (Zelezniak et al., 2015), the lack of antagonistic interactions between the Southern Ocean strains may be related to a sympatric speciation process by niche differentiation (Oakley et al., 2010), suggesting they would not compete for the same resources. However, this hypothesis should be confirmed by the metabolic characterisation of the interacting species. Furthermore, previous reports in *Streptomyces* suggest that the interactions between soil-derived strains tend to be reciprocal, which was particularly enhanced between strains isolated from the same soil sample (Vetsigian et al., 2011). This contrasts with the present results, as only the antagonistic interaction between *Pseudonocardia* sp. CNS-139 and *P. antitumoralis* were reciprocal. Only a few strains (*P. antitumoralis* DSM 45322, *P. sediminis* DSM 45779 and *Pseudonocardia* sp. CNS-139) showed two or more antagonistic interactions against other *Pseudonocardia* strains, while most of the combinations did not display any antagonistic interaction. These findings suggest that the free-living *Pseudonocardia* interacts with other members of the genus less often than their symbiotic counterparts, suggesting a broader ecological niche as expected.

Using mass spectometry imaging, we observed two metabolites (*m*/*z* 441.15 and *m*/*z* 332.20) that correspond to our antagonism assay results, as well as a metabolite (*m*/*z* 686.33) that was identified as a candidate to be a specialised metabolite produced by

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P. oceani KRD185 when the data are correlated with genomic data. This strategy was successfully applied before to analyse the role of several known metabolites in interactions involving *Streptomyces* (Traxler et al., 2013) and *Salinispora* (Patin et al., 2016). The metabolites of interest were detected with a higher intensity in interactions between geographically distant strains, suggesting that production is induced during microbial interaction. Similar results were described in *Pseudonocardia* strains related to fungus-growing ants when interacting with *Escovopsis*, where MSI allows the identification of ecologically relevant metabolites (Gemperline et al., 2017). We hypothesise that these metabolites could play a role in the interactions between the Southern Ocean *Pseudonocardia* strains. However, for the present work, the chemical structure of metabolites isolated from *P. abyssalis* KRD168 or *P. oceani* KRD185 has not yet been elucidated but will be the focus of future studies.

The reports of *Pseudonocardia* strains in symbiosis with fungus-growing ants showed that the antagonistic interactions observed in Petri dishes do not translate into a reduction of bacteria abundance on the cuticle of sub-colony ant workers (Poulsen & Currie, 2010). In the same way, it is possible to speculate that the antagonistic interactions observed between the marine-origin *Pseudonocardia* strains will affect the microbial populations in marine sediments. The specialised metabolites detected in the Southern Ocean *Pseudonocardia* strains could have other ecological roles, like nutrient use modulation. For instance, in *Streptomyces*, antibiotics in sub-inhibitory concentrations modulated nutrient use, minimising the niche overlap among competitors, which was proposed as an explanation for the low occurrence of antibiotic-resistant phenotypes (Vaz Jauri et al., 2013). Hence, the antagonistic interaction among the free-living *Pseudonocardia* cannot fully explain niche organisation in their natural environments. Furthermore, differences in the metabolic rate could affect the dynamic of the free-living *Pseudonocardia* in their natural environment. For example, a similar challenge assay performed in the genus *Salinispora* showed that the two closely related species *S. arenicola* and *S. tropica* can be differentiated based on their competitive strategies. The higher inhibitory activity occurred in the early stages for *S. arenicola*, while for *S. tropica* it occurred later, suggesting that they adapted different strategies to compete in marine sediments (Patin et al., 2016).

In summary, the challenge assay proved to be a successful strategy to modulate the production of metabolites by *Pseudonocardia* of marine origin. In particular, the combination of paired omics datasets, mass spectometry imaging, and challenge assays allowed the identification of a group of promising metabolites whose chemical identity and biological function await to be addressed in future work.

AUTHOR CONTRIBUTIONS

Jonathan Parra: Investigation; funding acquisition; writing – original draft; methodology; validation; visualization; formal analysis; data curation; conceptualization. **Scott A. Jarmusch:** Visualization; writing – original draft; methodology; data curation; investigation. **Katherine R. Duncan:** Conceptualization; funding acquisition; writing – review and editing; supervision; project administration.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Whole genome sequencing data were retrieved from the NCBI database; accession numbers are listed in Table S1. The data used for the molecular networking analysis were deposited in the GNPS-MassIVE repository under accession number MSV000091009: https://doi.org/doi:10.25345/C59W0982J. The MSI data have been made publicly available on METASPACE (https://metaspace2020.eu/project/parra-2023).

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