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Novel strains of Actinobacteria associated with neotropical social wasps (Vespidae; Polistinae, Epiponini) with antimicrobial potential for natural product discovery

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Abstract

Antimicrobial resistance has been considered a public health threat. The World Health Organization has warned about the urgency of detecting new antibiotics from novel sources. Social insects could be crucial in the search for new antibiotic metabolites, as some of them survive in places that favor parasite development. Recent studies have shown the potential of social insects to produce antimicrobial metabolites (e.g. ants, bees, and termites). However, most groups of social wasps remain unstudied. Here, we explored whether Actinobacteria are associated with workers in the Neotropical Social Wasps (Epiponini) of Costa Rica and evaluated their putative inhibitory activity against other bacteria. Most isolated strains (67%) have antagonistic effects, mainly against *Bacillus thuringensis* and *Escherichia coli* ATCC 25992. Based on genome analysis, some inhibitory Actinobacteria showed biosynthetic gene clusters (BGCs) related to the production of antimicrobial molecules such as Selvamycin, Piericidin A1, and Nystatin. The Actinobacteria could be associated with social wasps to produce antimicrobial compounds. For these reasons, we speculate that Actinobacteria associated with social wasps could be a novel source of antimicrobial compounds, mainly against Gram-negative bacteria.

Keywords: social wasps; adults; actinobacteria; inhibition; pathogen; antimicrobial compounds

Introduction

Antibiotics represent one of the most relevant breakthroughs of the 20th century for disease treatment. These chemical substances are produced mostly by microorganisms aiming to inhibit or delay the growth of pathogenic bacteria in their environment, as shown by the mold fungus *Penicillium chrysogenum* from which the first antibiotic was discovered over 70 years ago (Lima et al. 2020, Mann et al. 2021). Approximately, 70% of pathogenic bacterial species have evolved the ability to resist current standard antimicrobial treatments (Huddleston 2014, Bos and Austin 2018, Sharma et al. 2018, de Alcântara et al. 2020, Pérez et al. 2020), driven by the overconsumption of antibiotics. Therefore, common diseases and minor injuries could become deadly, threatening the global public health system (Sharma et al. 2018). The World Health Organization has issued the urgent need to find new targets and design new antibiotic compounds, mainly from sources that have not been explored (World Health Organization 2014, 2017, 2020).

Many organisms, including social insects, establish symbiotic relationships with antimicrobial producing microorganisms to prevent pathogen infections (Currie et al. 1999, Santos et al. 2004, Kaltenpoth et al. 2005, 2006, Poulsen et al. 2006, Stow and Bettie 2008, Kroiss et al. 2010, Graystock and Hughes 2011, Madden et al. 2013, Tranter et al. 2014, Matarrita-Carranza et al. 2017, Fukuda et al. 2021, Toninato de Paula et al. 2021, Schmidt et al. 2022, Turillazi et al. 2023). Social insects develop societies where thousands of individuals share a common space, the nest, where parasite colonization, growth, and dispersion are fostered by the specific conditions provided in social insects' nests including accumulation of larval waste, comfortable temperature, and retention of humidity (Jeanne 1991). For these reasons, social insects have evolved different antipathogen-defensive strategies, from nest-cleaning behavior to the establishment of symbiotic relationships, primarily with Actinobacteria, a phylum widely known by its production of antimicrobial secondary metabolites (Stow and Bettie 2008). According to Turillazi et al. (2023), Actinobacteria represent the most important group of microorganisms for the production of defensive substances in social insects. Fungus-growing ants (*Atta*, *Acromyrmex*), for example, maintain a symbiotic relationship with *Pseudonocardia* Actinobacteria to protect their fungal gardens from the pathogen *Escovopsis* (Currie et al. 1999, Stow and Bettie 2008). In addition, Actinobacteria have been isolated from other social insects including bees (Mohr and Tebbe 2006, Promnuan et al. 2009, Anderson et al. 2013, Corby-Harris et al. 2014, Toninato de

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Paula et al. 2021), termites (Rosengaus et al. 1998, Hamilton et al. 2011, Schmidt et al. 2022), and wasps (Madden et al. 2013, Matarrita-Carranza et al. 2017, Turillazi et al. 2023).

Social wasps are excellent models to perform exploratory studies on Actinobacteria, due to larval waste accumulation (meconia). Previous research reported actinobacterial presence in the adult cuticle and nest material of *Polistes dominulus* (Rosengaus et al. 1998, Kaltenpoth et al. 2005, 2006, Kroiss et al. 2010, Hamilton et al. 2011). Nevertheless, microorganisms associated with other groups of wasps such as the neotropical group Epiponini, have been scarcely studied (Matarrita-Carranza et al. 2017, Chavarría-Pizarro 2019). Epiponines could be a key group in the search for new antimicrobial substances since they accumulate high levels of meconia in their cells and reuse their brood cells, where immatures (egg, larvae, and pupae) develop on top of the meconia from previous larvae (Jeanne 1991). Consequently, there is more substrate for parasites to develop, demanding high production of antimicrobial substances to prevent pathogen infections in the colony members. Epiponini workers have an elaborate and meticulous cleaning behavior: when an immature completes its development and leaves the cell, workers and queens immediately insert their heads into the chamber (Jeanne 1991). Adults could transmit antimicrobial substances to sterilized cells as has been observed in other insects, like ants that secrete antibiotics from glands located in legs and mandibles (Brough 1983, Veal et al. 1992, Li et al. 2018), and *Philanthus* wasps, which host Actinobacteria in the antenna (Kaltenpoth et al. 2005, 2006, Kroiss et al. 2010). This behavioral observation could indicate high levels of parasite development in Epiponini colonies, favored by the excess of meconia accumulated in their cells.

We hypothesized that Actinobacteria are associated with workers in the Neotropical Social Wasp Epiponini, as it has been previously found in other groups of Hymenoptera. Therefore, we analyzed the cuticle and salivary glands of adult workers from several species from three of the four main clades of Epiponini. Our main objectives were: (1) to isolate Actinobacteria strains from each of the Epiponini species; (2) to identify Actinobacteria associated with Epiponini at the molecular level; (3) to evaluate Actinobacteria inhibitory activity against pathogens related to insect and human infections; and (4) to evaluate their genetic potential as a source of natural products. To our knowledge, this is the first study that assesses Actinobacteria associated with several morphological species of Epiponini ($N = 13$) and evaluates their inhibitory properties.

Material and methods

Adult wasp collection

Adult samples were collected from different species of Epiponini. Neotropical social wasps of Epiponini (Hymenoptera: Vespidae; Polistinae) include 19 genera distributed from the southern USA to Argentina. Epiponini species have a high degree of social organization like ants and honeybees (eusocial insects), characterized by: swarm-founding, worker labor division, and different morphological caste syndromes, among other traits. To obtain representative samples, we collected individuals from colonies of different genera that represented three of the four main clades of Epiponini (Noll et al. 2020) as described: clade 2: *Parachartergus* and *Chartergellus*; clade 3: *Protopolybia*; and clade 4: *Polybia* and *Metapolybia* (Fig. 1).

Adult samples were collected in four regions of Costa Rica: a tropical dry forest in the North Pacific (Santa Cruz, Guanacaste), an urban area in Central Valley (Cartago), a wet lowland tropical forest in the Atlantic (La Selva in Sarapiquí, Heredia), and South Pacific (Golfito, Puntarenas). First, a sample of 20 adults (workers) from each nest (Table 1) was collected with sterile forceps, placed into sterile and hermetic bags, and stored at 4°C in a cooler for transportation (Madden et al. 2013). Then, sterile bags were placed in a freezer at −15°C to cold-immobilized workers (Hamilton et al. 2011). At the moment of transportation to the laboratory, bags were placed again in the cooler. The samples were stored at −20°C for no longer than 15 days before processing (Hernández and Cafaro 2015) in the Biotechnology Research Center (CIB) from Instituto Tecnológico of Costa Rica. *Polybia* was the most abundant genera and we obtained adults from eight colonies ($N = 128$) from three regions (40 adults from Cartago and Guanacaste, 80 from Golfito) (Table 1). From *Parachartergus* we obtained 64 adults from two regions, and from *Metapolybia* and *Protopolybia*, which were more challenging to find, we obtained 40 adults only from one region (Table 1). From Guanacaste and Golfito we sampled an equal number of adults ($N = 96$), from Cartago we sampled 64 adults, and from La Selva, we sampled a lower number of adults ($N = 16$) because access was limited due to pandemic restrictions (since March 2020). Details of adult collection from each sampling region are summarized in Table 1.

Actinobacteria isolation from wasps

To obtain Actinobacteria strains from wasps, we followed Madden et al. (2013) and Hamilton et al. (2011) protocols. One worker of each colony was used to inoculate each culture medium (x4): (a) 1% Luria–Bertani (LB; Miller) medium; (b) Actinobacteria isolation agar for microbiology (Sigma-Aldrich); and (c) International Streptomyces Project 1 and 2 (ISP1 and ISP2) media. Each medium was supplemented with nystatin (0.1%) and nalidixic acid (10 ug/ml) to reduce the growth of microorganisms different from Actinobacteria. To isolate Actinobacteria from the cuticle, every individual was washed with phosphate buffered saline and then rubbed across the entire surface plate with sterilized forceps, assuring that all insect parts touched the plate. We used one individual of each colony per culture media plate, and we performed two inoculations per colony, as a consequence we used a total of eight individuals to obtain Actinobacteria from the cuticle. To isolate microorganisms from salivary glands, we used different individuals from those used for the cuticle, we used one individual of each colony per culture media plate, and we performed two inoculations per colony. We extracted salivary glands situated in the thorax with sterilized entomological forceps and needles in a sterilized environment. Because of their location in the wasp body, salivary glands were easy to extract without touching or scraping another structure (like the foregut). Dissected glands of every individual were homogenized by vortexing in 30 ul of 100 mM sodium acetate (pH 5) for 15 seconds. Then, 20 ul of the homogenized solution of every individual were plated in each medium. After inoculation, the plates were incubated at room temperature (~22°C) for ~1 month. To verify that growing microorganisms were obtained from wasps and were not the result of contamination during sampling or preparation, a control plate of each culture media with no inoculum was placed next to all inoculated plates. Colonies with Actinobacteria-like morphology (i.e. filamentous, Gram-positive bacteria) were selected for further purification and preservation at −70°C in 20% glycerol.

Molecular identification of Actinobacteria

To identify Actinobacteria strains isolated from wasps, genomic DNA was obtained by modifying a CTAB-based method according to Perez et al. (2017) and Chun and Goodfellow's (1995)

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Figure 1. Social wasp nests of *Polybia* sp. (A) and (B), *Metapolybia* sp. (C), *Chartergellus golfitensis* (D), and *Protopolybia* sp. (E) selected for sample collection.

protocol. Briefly, DNA was obtained from 1 mg of each Actinobacteria colony. Bacterial material was washed with sterile distilled water (1 ml) and centrifuged at 14 000 rpm (maximum speed) for 7 minutes. Subsequently, we repeated the previous procedure but using STE solution (100 mM NaCl + 25 mM Tris + 10 mM EDTA). Then, the supernatant was discarded, and the pellet was resuspended in 400 µl of STE with 20 µl of lysozyme solution (50 mg/ml). After overnight incubation at 37ºC, 100 ul of 20% SDS was added, vortexed for 1 minute, and incubated inside a 55°C water bath for 1 hour. Then, 100 µl of 3 M sodium acetate and 500 µl of phenol/chloroform/isoamyl alcohol (25:24:1) were added, mixed by inversion, and centrifuged at 14 000 rpm (maximum speed) for 5 minutes. The upper aqueous phase was transferred to another 1.5 ml vial, and an equal volume (~650 µl) of chloroform/isoamyl alcohol (24:1) was added, mixed by inversion, and centrifuged for 10 minutes at 14 000 rpm (maximum speed). The upper phase was transferred to a new 1.5 ml vial, and an equal volume of cooled isopropanol (~500 µl at −20°C) was added. Finally, it was centrifuged at 14 000 rpm (maximum speed) for 7 min, the supernatant was discarded, and the pellet was washed with cooled 70% ethanol (1 ml at −20°C). Then, the DNA was resuspended in 50–100 ul of TE buffer (10 mM Tris-HCl + 1 mM EDTA) with 1 µl of RNAase (10 mg/ml) and stored at −20°C.

DNA samples were sent to Psomagen for 16S rRNA gene amplification and sequencing, using universal eubacteria primers 27F and 1492R. Obtained sequences were trimmed and consensus (Madden et al. 2013), the contiguous sequence was created using Snapgene Viewer® software and was alignment using Clustal. We then compared with GenBank public nucleotide sequence database using BLAST with default parameters (Altschul et al. 1990), specifying 16S rRNA gene region for prokaryotic organisms.

Antimicrobial activity tests

Inhibition of bacterial growth was assessed against strains from three bacterial species, which harbor known human pathogens: *Escherichia coli* (strain ATCC 25992), *Pseudomonas aeruginosa* (strain ATCC 9027), and *Staphylococcus aureus* (strain ATCC 25923), all obtained from the Microbiology Department of Universidad de Costa Rica. We also assessed growth inhibition against the insect pathogen *Bacillus thuringensis* from the collection of Biocontrol Laboratory of CIB-ITCR (code Bt1 isolated from *Phyllophaga* sp). Briefly, 0.2 ml of 24-hour liquid culture of tested bacteria was inoculated spreading on a nutrient agar plate. Finally, a fragment of 10 mm diameter from Actinobacteria culture media (incubated for 3 weeks as described above) was placed on the inoculated plate. The inhibition halo was measured after 24 hours of incubation at 37ºC. Kanamycin (KM) at 6 mg/ml was used as a positive control for assays with *P. aeruginosa*, *B. thuringensis*, and *S. aureus*; and at 5 mg/ml for assays with *E. coli* on a 5-mm disk.

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Table 1. Information of wasp nests employed to collect workers from four regions of Costa Rica.

Wasp genus	Nest code	Location	Data
Polybia sp1	PbSB-01	SB*	20/04/2019
Polybia sp3	PbStC-02	SB	28/09/2019
Metapolybia sp1	MSB-01	SB	08/06/2019
Metapolybia sp1	MSB-02	SB	28/09/2019
Parachartergus sp2	MPchSB-02	SB	02/08/2019
Parachartergus sp2	PchN-02	SB	27/09/2019
Parachartergus sp1	MPchC-01	ITCR@	19/07/2019
Parachartergus sp1	IPchC-01	ITCR	02/12/2020
Polybia sp2	PbC-01	ITCR	31/07/2019
Polybia sp2	IPbC-01	ITCR	02/12/2020
Protopolybia sp1	PrtSel-01	OET**	20/07/2019
Polybia sp4	PbG-01	RFSGΨ	30/01/2020
Polybia sp5	PbG-01	RFSG	30/01/2020
Protopolybia sp2	PrtG-01	RFSG	30/01/2020
Polybia sp6	PbG-03	RFSG	19/03/2021
Polybia sp7	PbG-04	RFSG	19/03/2021
Chartergellus golfitensis	ChG-01	RFSG	19/03/2021

*SB = Santa Bárbara, Santa Cruz, Guanacaste. 0°10′23.7″ N, 85°35′38.5″ W; rural area at 144 m above sea level, annual average temperature (27°C) and rainfall of 765 mm (IMN 2021).
@ITCR = Instituto Tecnológico de Costa Rica, Cartago. 9°51′17.9″ N, 83°54′33.9″ W; urban area at 1435 m above sea level, annual average rainfall of 1500–2000 mm, and temperature 12–20°C (IMN 2021).
**OET = La Selva, Sarapiquí, Heredia. 10°25′19″ N, 84°00′54″ W; rural area at 35 m above sea level, annual temperature between 19–31°C, and annual average rainfall of 4000 mm (IMN 2021).
ΨRFSG = Refugio de Fauna Silvestre Golfito 8°39′15.8″ N, 83°10′45.1″ W; rural area at 62 m above sea level, an annual amount of precipitations 4000 mm and temperature 25°C (IMN 2021).

Sterile distilled water was used as a negative control. Assays were performed in duplicate, and inhibition of tested bacterial growth was analyzed by comparison to the positive control. Inhibition of pathogen growth was considered negative when no halo surrounding the Actinobacteria was observed. Results obtained from replicates were averaged.

Genome sequencing and assembly

Six assembled genomes were obtained from a sample of the strains that inhibited most of the bacterial strains that harbor known human pathogens and presented greater inhibition halos (6M, 6T, 6V, 7B, 7G, 8L) in order to search for biosynthetic gene clusters (BGCs) related to the production of antibacterial molecules. Genomic libraries were prepared with Nextera XT DNA Library Preparation kit (Illumina) and sequenced using a paired-end strategy of 2 × 150 nucleotides on the Illumina NextSeq500 platform (Illumina, San Diego, USA). Raw sequencing reads were quality controlled and trimmed using fqCleaner (https://gitlab.pasteur.fr/GIPhy/fqCleanER) with all the “-s” options activated and clipping of Illumina Nextera index. *De novo* assemblies were obtained from trimmed reads using SPAdes 3.14.1 (Bankevich et al. 2012) with options: -k 21,33,55,77 –only-assembler –careful. The quality of assembled genomes was measured with CheckM v1.1.3 (Parks et al. 2015). Default parameters were used for data processing except where otherwise noted. Further details about the obtained sequences and genome assemblies were reported in Gutiérrez-Araya et al. (2022) and are available in the DDBJ/ENA/GenBank repository under the Bioproject accession number PRJNA764377 (genome assemblies: GCA_020176775.1, GCA_020177455.1, GCA_020176785.1, GCA_020177435.1, GCA_020177475.1, and GCA_020176795.1).

Multilocus phylogenetic analysis

A phylogenetic analysis was performed to confirm the taxonomic assignment and determine the evolutionary relationship between the selected strains and the closest taxa. Reference genomes from each genus were obtained from GenBank and run on autoMSLT (Automated Multi-Locus Species Tree) (https://automlst.ziemertlab.com) with predetermined parameters. Briefly, for the construction of this phylogenetic tree, HMMER selects homologous genes from the genomes, then the orthologous genes with conserved functions are confirmed with TIGRFAM. The resulting list of genes is prioritized with the precalculated dN/dS values and a maximum of 100 genes is selected. The selected single-copy genes were then aligned and placed in the tree using MAFFT and the RAxML Evolutionary Placement Algorithm. Finally, a maximum-likelihood tree was built with 1000 ultrafast bootstrap and IQ-TREE automatic model search. The inference of the definitive species tree was carried out with ASTRAL-III (Zhang et al. 2018). Additionally, a taxonomic comparison based on average nucleotide identity (ANI) was made using fastANI against the closest reference complete genomes available on Genbank (n = 26 for *Shaccharopolyspora* and n = 376 for *Streptomyces*).

Functional and biosynthetic gene prediction

Genomic Annotation was performed with RAST (Rapid Annotation Subsystem Technology) server using the default parameters (Aziz et al. 2008). For secondary metabolism functional annotation, AntiSMASH 5.0 software was used. BGC prediction was performed with default parameters in relaxed mode and activation of the following features: MIBiG cluster comparison, Pfam cluster analysis, cluster BLAST, active site finder, subcluster BLAST, RREFinder, and TIGRFam analysis (Blin et al. 2019). Strains were clustered based on their genus and classification of the BGCs was performed according to the following types: Lanthiopeptide-class-ii; RiPP; Rantiopeptide; NRPS, Terpenes, T1PKS, T2PKS, Arylpolyene, CDPS, NAAPAA, TransAT-PKS, Ectoin, Butyrolactone, Lanthiopeptide Class iii, Peptide Loop, RRE, and Anglicide.

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Table 2. Number and identity of Actinobacteria strains isolated from workers of Neotropical social wasps (Epiponini) from different locations.

Closest taxon based on 16SrRNA BLAST	Guanacaste	Cartago	La Selva	Golfito	Total
Saccharopolyspora	22	0	1	1	24
Streptomyces	0	4	1	2	7
Amycolaptosis	6	0	0	0	6
Microbacterium	2	2	0	0	4
Nocardiopsis	3	0	0	0	3
Kocuria	1	1	0	0	2
Tsukamurella	0	0	0	2	2

Results

Identification of Actinobacteria obtained from wasps

We sampled adult wasps from several genera and from different ecological environments in Costa Rica to investigate the presence of associated microorganisms. A total of 48 strains of Actinobacteria were obtained from workers' cuticles and salivary glands. Due to differential sampling of wasps' nests, the genera with the highest number of strains belonged to *Polybia* ($N = 23$), followed by *Parachartergus* ($N = 20$), and *Metapolybia* ($N = 16$). The region with the highest number of isolated strains was Santa Cruz ($N = 35$), which represents an example of a tropical dry forest, followed by Cartago ($N = 7$), an urban area, and Golfito ($N = 6$), a South Pacific wet lowland tropical forest.

According to the 16S rRNA gene BLAST search, Actinobacteria strains had high similarity (between 95% and 97%) with eight genera (Table 2). Most strains were identified as *Saccharopolyspora* ($N = 24$), *Streptomyces* ($N = 7$), and *Amycolaptosis* ($N = 6$). Strains of *Amycolaptosis* and *Nocardiopsis* were obtained only from Santa Cruz wasps. *Tsukamurella* strains were obtained only from Golfito. On the other hand, *Saccharopolyspora* was isolated from workers in all regions except Cartago; *Streptomyces* was not obtained from workers in Santa Cruz, and *Microbacterium* and *Kocuria* were not isolated from workers in La Selva and Golfito.

Most of the strains were isolated from the cuticle ($N = 39$), and only 10 strains were obtained from salivary glands. *Microbacterium* strains were obtained only from salivary glands; *Kocuria*, *Saccharopolyspora*, and *Streptomyces* were obtained from both glands and cuticles. On the other hand, *Amycolaptosis*, *Nocardiopsis*, and *Tsukamurella* were obtained only from workers' cuticles.

Inhibition of microorganisms by wasp-associated Actinobacteria

We investigated the inhibition of *E. coli*, *P. aeruginosa*, *S. aureus*, and *B. thuringensis*. We found that most of the isolated strains, 30 of the 49 obtained (61%), inhibited tested bacterial growth. For four Actinobacteria strains (8%), it was not possible to obtain enough biomass (because of limited growth *in vitro*) to perform inhibition tests; and only 12 strains (25%) did not inhibit the growth of any tested bacteria.

Most of the strains ($N = 29$) that inhibited tested bacteria growth ($N = 30$) showed antagonistic effects against *B. thuringensis*; strains that showed higher inhibition halo, related to antibiotic control, were *Nocardiopsis*-P1A, *Amycolaptosis*-P1P and P1S, *Saccharopolyspora*-6A, *Microbacterium*-8 G, and *Streptomyces*-8 L (Table 3). Most of the inhibitory strains were obtained from Guanacaste ($N = 19$) and were isolated from adults of *Metapolybia* ($N = 9$), *Polybia* ($N = 9$), and *Parachartergus* ($N = 8$) (Table 3).

Similarly, 25 strains of the total of strains that inhibited tested bacteria ($N = 30$), had antagonistic effects against *E. coli* growth. The strains showing higher inhibition halos against *E. coli* were *Nocardiopsis*-P1A, *Amycolaptosis*-P1P, *Saccharopolyspora*-6T, and 6 V, and *Streptomyces*-7 G (Table 3). Most of the strains that inhibited *E. coli* were obtained from Guanacaste ($N = 19$), corresponding to strains that also inhibited *B. thuringensis*.

In the case of *P. aeruginosa*, 19 of the total antagonistic Actinobacteria strains ($N = 30$) inhibited it. Strains with higher inhibition halos were *Streptomyces*-MP and 8 L, and *Saccharopolyspora*-6 W (Table 3). Most of the inhibitory strains were obtained from Guanacaste ($N = 18$), mostly from adults of *Metapolybia* ($N = 9$), *Polybia* ($N = 9$), and *Parachartergus* ($N = 5$).

On the other hand, isolated strains showed weak inhibition against *S. aureus*. Only nine of all antagonistic strains ($N = 30$) formed an inhibition halo (Table 3). Half of the strains that inhibited *S. aureus* were isolated from Guanacaste; all were obtained from *Parachartergus*. The strains that showed inhibition against all tested bacteria were *Streptomyces* (7 G, MA), *Amycolaptosis* (P1P, P1S), one *Saccharopolyspora* (8S), and one *Nocardiopsis* (P1A) (Table 3).

Genome-based analysis of pathogen-inhibiting strains

Six assembled genomes were obtained from strains displaying antibacterial activity: four *Saccharopolyspora* sp. (6M, 6T, 6V, and 7B) and two *Streptomyces* sp. (7G and 8L). Genomes ranged from 6.4 to 9.0Mb and were previously reported (Gutiérrez-Araya et al. 2022). Genome annotation showed that our strains display a variable amount of CDSs (Fig. 2), even though the ANI results showed that all four *Saccharopolyspora* strains seem to belong to the same species (Gutiérrez-Araya et al. 2022). Genes related to the production of amino acids, carbohydrates, vitamins, prosthetic groups, and pigments were the most abundant. Moreover, all the strains have subsystems related to secondary metabolism and plasmids.

Taxonomic phylogenetic analysis based on core genes confirms the close relationship among the *Saccharopolyspora* strains. Those strains were found in a separate clade sharing an ancestral node with *S. gloriosae*. However, *S. gloriosae* is below the species threshold, as confirmed by an ANI of 84.56%. These results indicate that *S. gloriosae* is the closest taxonomic group to our wasp-associated bacteria, but these probably belong to a novel species classification according to their whole genome similarity (Fig. 3).

Strains 8L and 7G (Fig. 4) were found in different clades. ANI results confirm the taxonomic distance between those strains, sharing only 76% genomic identity, indicating that both strains belong to the *Streptomyces* genus but are distant species. The phylogenetic tree shows that strain 8L groups with *Streptomyces* sp. *S4.7* and *S. niveus* SCSIO 3406, but in a different subclade. Moreover,

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Table 3. Growth inhibition halos formed by Actinobacteria strains obtained from workers of different Neotropical social wasps (Epiponini), tested by disk diffusion assay against known bacterial pathogens.

			Inhibition halo (mm)			
Wasp genus	**Actinobacteria**	**Strain code**	***B. thuringensis***	***E. coli***	***S. aureus***	***P. aeruginosa***
Polybia	*Saccharopolyspora*	6A	31 ± 1.4	22 ± 2.1	0	0
Polybia	*Saccharopolyspora*	6B	19 ± 5.7	15.5 ± 0.7	0	0
Polybia	*Saccharopolyspora*	6G	28 ± 0	13.5 ± 2.1	0	0
Polybia	*Saccharopolyspora*	6I	19.8 ± 1	19 ± 0	0	14.5 ± 0.7
Polybia	*Saccharopolyspora*	6J	23 ± 0	15.5 ± 0.7	0	0
Polybia	*Saccharopolyspora*	6K	25 ± 0	21 ± 0.7	0	0
Metapolybia	*Saccharopolyspora*	6M	26.5 ± 0.7	22.5 ± 2.1	0	19.5 ± 0.7
Metapolybia	*Saccharopolyspora*	6N	30.5 ± 2.1	19 ± 0	0	0
Metapolybia	*Saccharopolyspora*	6O	21.5 ± 0.7	15 ± 0	0	0
Metapolybia	*Saccharopolyspora*	6T	19.8 ± 0.4	24 ± 1.4	0	21.5 ± 0.7
Metapolybia	*Saccharopolyspora*	6U	22 ± 1.4	12.5 ± 3.5	0	22.5 ± 0.7
Metapolybia	*Saccharopolyspora*	6V	21.5 ± 0.7	24 ± 0.7	0	22.5 ± 2.1
Metapolybia	*Saccharopolyspora*	6W	29 ± 1.4	20 ± 0	0	30.5 ± 0.7
Metapolybia	*Saccharopolyspora*	6Z	30.5 ± 0.7	21.5 ± 2.1	0	0
Metapolybia	*Nocardiopsis*	7B	28 ± 2.8	20 ± 0	0	20 ± 0
Protopolybia	*Saccharopolyspora*	7F	21.5 ± 2.1	0	0	0
Protopolybia	*Streptomyces*	7G	24 ± 1.4	24 ± 1.4	11.5 ± 0.7	12 ± 0
Parachartergus	*Microbacterium*	8G	31 ± 1.4	16.5 ± 0.7	11 ± 0	0
Polybia	*Tsukamurella*	8F	29 ± 0	18.5 ± 3.5	0	0
Polybia	*Tsukamurella*	8J	16.3 ± 0.3	20 ± 0	0	23 ± 0
Protopolybia	*Streptomyces*	8L	31 ± 0	22.5 ± 0.7	0	30 ± 0
Polybia	*Saccharopolyspora*	8S	25.2 ± 0.4	18.3 ± 0	15.3 ± 0.7	16.3 ± 0
Parachartergus	*Streptomyces*	MA	16 ± 0.7	20 ± 0	16 ± 0.7	17 ± 0.7
Parachartergus	*Kocuria*	MB	17 ± 0.7	0	0	20 ± 0
Parachartergus	*Microbacterium*	MN	0	0	0	24 ± 0.4
Parachartergus	*Microbacterium*	MO	21 ± 0.4	0	15 ± 0.7	16 ± 0.7
Parachartergus	*Streptomyces*	MP	22 ±	0	19 ±	35 ± 1
Parachartergus	*Nocardiopsis*	P1A	37 ± 0.7	27 ± 0.4	30 ± 1	24 ± 0.4
Parachartergus	*Amycolaptosis*	P1P	35 ± 1	24 ± 0.4	29 ± 0.7	24 ± 0.4
Parachartergus	*Amycolaptosis*	P1S	35 ± 1	23 ± 0.4	28 ± 0	19 ± 0.4
Kanamycin control			38.3	28.3	29.3	27.3

comparing this strain against 376 *Streptomyces* genomes, ANI values range from 78.1% to 81.4%. Therefore, 8L has no match at the species level with other complete genomes of *Streptomyces* (species threshold < 95%). Phylogenetically closest species have not been previously associated with insects.

On the other hand, strain 7G shares a clade with other *Streptomyces* species, including *S. globisporus* and *S. mediolani* (Fig. 3). ANI results confirmed the identity of 96% with two strains of *S. globisporus* isolated from soil and tomato flower (accession numbers GCF_003147545.1 and CF_000261345.2). In comparison, values higher than > 95% were found with *Streptomyces* sp. WA6-1-16 isolated from *Periplaneta americana* intestine, *Streptomyces* sp. Tue 6075 from soil forest and *Streptomyces* sp. R527F from Arctic Ocean sediment. Therefore, the results suggest that 7G belongs to the species *S. globisporus*.

Genomic functional annotation: prediction of BGCs

We identified BGCs associated with antimicrobial compounds in all the isolates from Epiponini wasps. For instance, in the *Saccharopolyspora* sp. strains, 17 types of BGCs were identified. The most common BGCs were classified as NRPS, Class 1 Polyketides (T1PKS), and Terpenes (Table 4). Five BGCs showed high similarity (> 60%) to the genes related to the synthesis of Geosmin, Gyralimycin 1a, Ery-9/Ery-6/Ery-8/Ery-7/Ery-5/Ery-4/Ery-3, Iso-migrastatin, SGR PTMs, and Nystatin A1. On the other hand, 15 gene clusters showed moderate genetic similarity (30%–59%) to Herboxydiene, Cinamycin, Ery-9, Iso-migrastatin, Catelecin, Tetracenomycin C, Anantin C, Thiazostatin, Telomycin, Isorenieratene, Brasilicardin A, Nystatin, Piericidin A1, Selvamycin, and Lasalocid clusters. A total of 13 BGCs showed low similarity (2%–29%) to known clusters for Pyralomicin 1a, Herboxidiene, Thaxteramide C, Polyoxin A/H, Thacteramide C, Thiazostatin, Telomycin, Bacillibactin, CDA1b Coelibactin, SF2575, Carotenoid, and Prejadomycin. Finally, seven BGCs classified as Butyrolactone, Ranthipeptide, NRPS, and T1PKS did not show genetic similarities with any cluster on the database.

The diversity of BGCs was higher for strains of the genus *Streptomyces* than that found on *Saccharopolyspora* sp. (Table 5). In this case, 44 gene clusters were predicted in the strain 7G, while 36 were found in the strain 8L. Those BGCs were classified into 18 different types. The highest number belongs to NRPSs (26%), followed by T1PKS (16%) and terpenes (13%). We obtained 15 predicted BGCs with high similarity (> 60%) to the known gene clusters of ROS 12–3108, AmfS, Keywimisin, Melanin, Hedamycin, Ectoin, Alquilerorcinol, Besderrioxamine B, SGR PTMs, Methylisoborneol, Geosmin, Isorenieratene, Hopene, and Coeliquelin. Three BGCs showed moderate similarity (30%–59%) to Lasalocid, Celibactin, and Streptobactin. Most BGCs showed low similarity (2%–29%) with Methylenomycin A, Showdomycin, Pepticinnamin E, Kanamycin, Herboxidiene, Coelimycin P1, SF2575, RP-1776, Ficellomycin, Akaeolide, Lagunapyrone A, and Enduracidin. On the other hand, HgIE-KS, RRE-containing, Lanpeptide-clase-I, RiPP-like, Butyrolactone, Siderophore, T1PKS, Terpene, and NRPS gene clusters did not match with any other known BGCs (Table 5).

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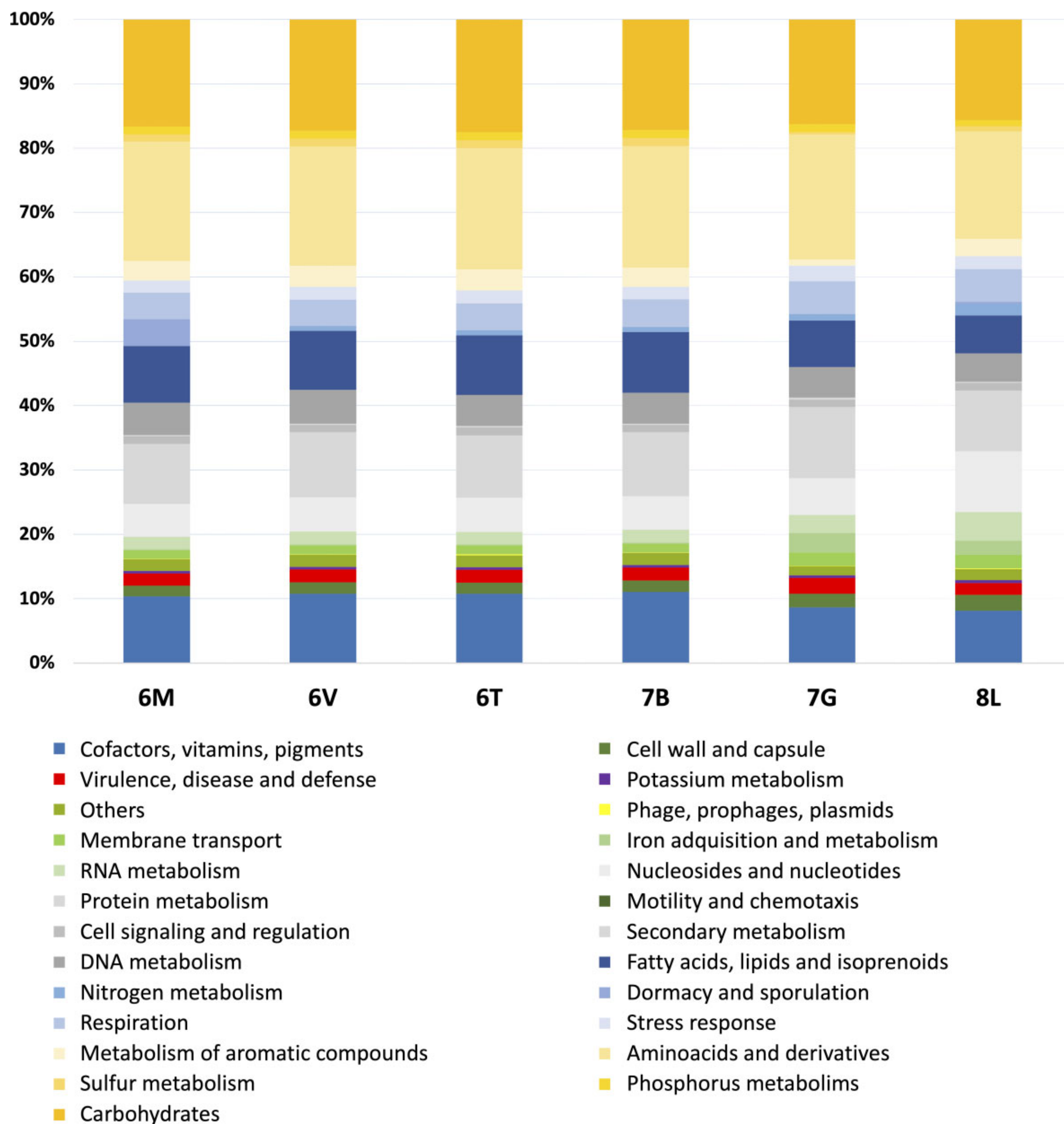

Figure 2. Distribution of coding sequences based on the genome annotation and categorized in subsystems for six strains of wasp-associates actinobacteria (*Saccharopolyspora* sp. 6M, 6T, 6V, 7B, and *Streptomyces* sp. 7G and 8L).

Discussion

To our knowledge, this is the first study that isolated Actinobacteria from salivary glands (not only from the cuticle of adult wasps), obtained from five different Epiponini genera and 13 morphological species, collected from four geographic regions of Costa Rica (not only one as Matarrita-Carranza et al. 2017, 2021). Additionally, this is the first study that tested the antimicrobial inhibition of seven Actinobacteria genera against three bacterial species that harbor known human pathogens and included a genomic evaluation of Epiponini-associated Actinobacteria to identify promising sources for antibiotic compounds.

Social wasps are associated with Actinobacteria with inhibitory activity

Actinobacteria could provide multiple advantages for insects' survival in a wide range of habitats, as they require few resources due to their slow growth, use different substrates, and exhibit high metabolic versatility (Kaltenpoth 2009). For example, Actinobacteria strains were not only associated with Epiponini workers through their cuticles as previously observed (Matarrita-Carranza et al. 2017), but they are also present in salivary glands. In addition, some wasps-associated Actinobacteria strains could be specific to a geographic region. It has been suggested that Actinobacteria's presence in an environment depends on ecosystem characteristics such as plant species, soil composition, and diversity of other microorganisms (Kaltenpoth 2009, Govindasamy et al. 2014, Singh and Dubey 2018). Microhabitat conditions of Guanacaste ecosystems might favor the establishment of Actinobacteria like *Nocardiopsis*, *Amycolaptosis*, and *Saccharopolyspora*, in the environments (plants, soil, and so on) visited by social wasp adults. Similarly, the environmental conditions of Golfito could favor *Tsukamurella* strains that were isolated only from lowland rainforest wasps. Strains belonging to *Streptomyces*, *Microbacterium*, and *Kocuria* were found in multiple sampled environments. Although more studies should be carried out to understand actinobacterial

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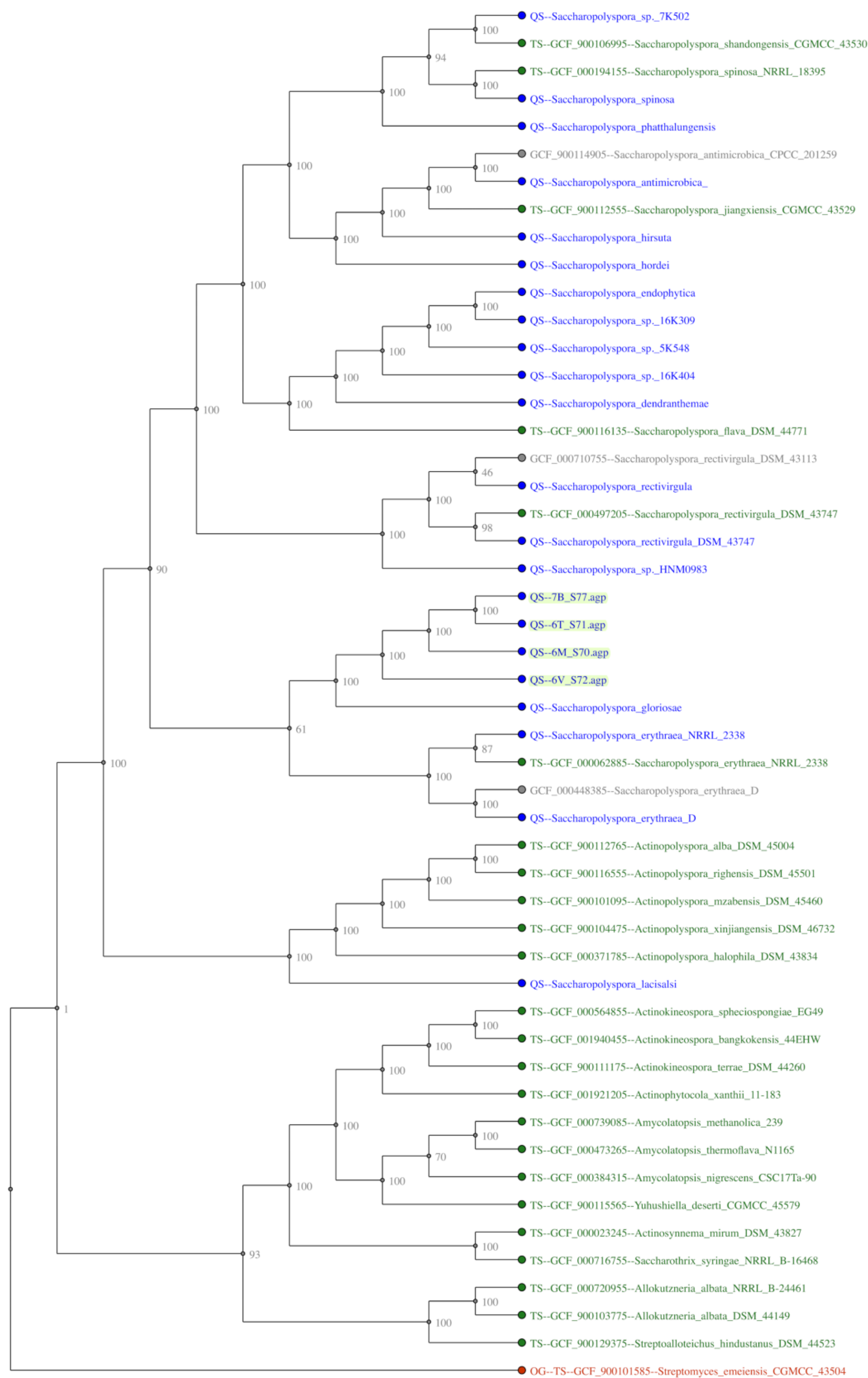

Figure 3. Maximum-likelihood phylogenetic tree based on the core genome of the isolated strains of the genus *Saccharopolyspora* (7B, 6M, 6V, and 6T, highlighted) and reference genomes selected from the NCBI GenBank database (identified as "QS" in blue), the automatically generated type genomes in autoMLST (identified as "TS" in green and gray), and the tree ancestor or "out-group" (identified as "OG" in red). Bootstrap support is indicated on the nodes and was obtained with 1000 replicates on the ultrafast method.

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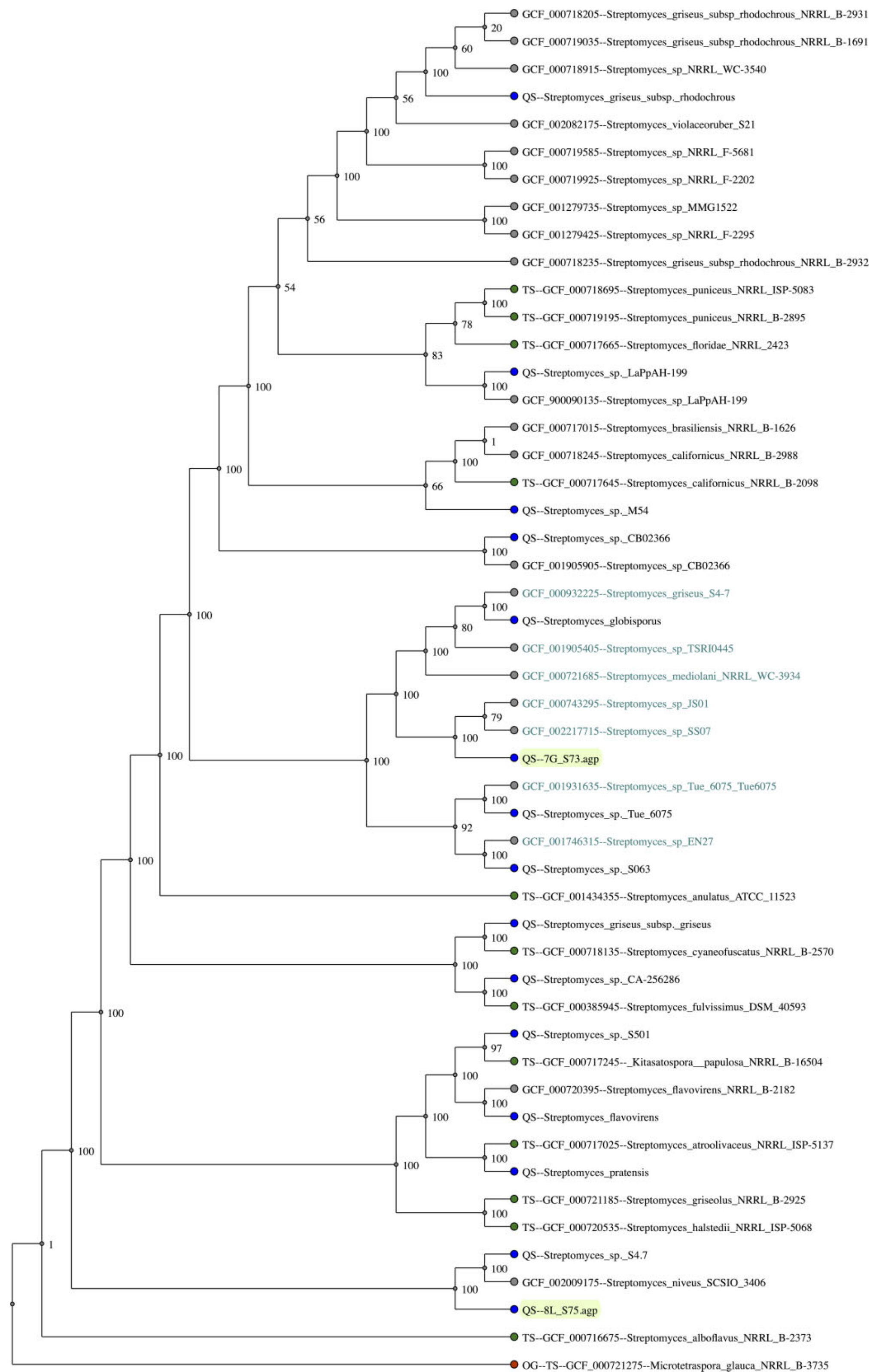

Figure 4. Maximum-likelihood phylogenetic tree based on core genomes of the *Streptomyces* isolates (7G and 8L, highlighted) and reference genomes selected from the NCBI GenBank database (identified as "QS" in blue), the type genomes generated automatically in autoMLST (identified as "TS" in green and gray), and the out-group (identified as "OG" in red). Bootstrap support is indicated on the nodes and was obtained with 1000 replicates on the ultrafast method.

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Table 4. Summary of BGC types identified in the genome of *Saccharopolyspora* strains (6M, 6T, 6V, and 7B) and their similarity to known BGCs according to the analysis performed with the AntiSMASH bioinformatics tool.

Predicted BGC type	Known BGCs similarity (percentage of matching genes)
Amglicyicl	Pyralomicin 1a (18%)
Arylpolyene and CDPS	Herboxidiene (38%), (2%)
Butyrolactone	N/A
Ectoine	Ectoine (100%)
Lantipeptide-class-ii	Cinnamycin (52%), (38%)
Lantipeptide-class-iii	Ery-9–Ery-6–Ery-8–Ery-7–Ery-5–Ery-4–Ery-3 (100%), (50%)
Lassopeptide	Iso-migrastatin—Migrastatin—Dorrigocin A—Dorrigocin B–13-Epi-dorrigocin A (54%), (45%)/Cattelecin (50%)
T2PKS	Tetracenomycin C (33%)
TransAT-PKS	Iso-migrastatin—Migrastatin—Gorrigocin A—Gorrigocin B–13-Epi-dorrigocin A (63%), (54%), (45%)
NAPAA	Thaxteramide C (7%)/Polyoxin A/Polyoxin H (5%)/Thacteramide C (7%)
Ranthipeptide	N/A
RiPP-like	Cinnamycin (52%) y (38%)
RRE-containing	Ery-9–Ery-6–Ery-8–Ery-7–Ery-5–Ery-4–Ery-3 (100%)/Anantin C (50%)
NRPS	SGR PTMs (66%)/Thiazostatin—Watasemycin A—Watasemycin B–2-hydroxyphenylthiazoline enantiopyochelin—isopyochelin (40%), (26%), (20%)/Telomycin (38%), (23%)/Bacillibactin (15%)/3 CDA1b (22%)/Coelibactin (18%)/1 N/A
Terpene	Geosmin (100%) y (60%)/Isorenieratene (57%)/Brasilicardin A (38%)/Hopene (30%)/SF2575 (6%)/Carotenoid (18%)/Prejardomycin (4%)
T1PKS	SGR PTMs (66%)/Nystatin A1 (63%) y 1 (36%)/Nystatin (57%)/Piericidin A1 (50%), (33%)/Selvamicin (33%)/lasalocid (36%)/4 N/A

N/A = no matching.

Table 5. Summary of biosynthetic cluster types (BGCs) identified in the genome of *Streptomyces* genus strains (7G and 8L) and their percentage similarity to known BGCs according to the analysis performed with the AntiSMASH bioinformatics tool.

Predictied BGC type	Known BGCs similarity (percentage of matching genes)
Furan	Methylenomycin A (28%)
hgIE-KS	N/A
Lantipeptide-class-ii	SRO 12–3108 (100%)
Lantipeptide-class-iii	AmfS (60%)
Lassopeptide	Keywimisin (80%)
Melanin	Melanin (100%)
Nulcleosido	N/A
RRE-containing	N/A
T2PKS	Hedamicine (87%)
Ectoine	Ectoine (100%)/Showdomycin (23%)
Lantipeptide-class-i	Pepticinnamin E (10%)/N/A
RiPP-like	Kanamycin (1%)/4 N/A
T3PKS	Alkylerorcinol (100%)/Herboxidiene (5%)
Butyrolactone	Coelimycin P1 (16%)/SF2575 (6%)/RP-1776 (6%)/and 1 N/A
Siderophore	Desferrioxamin B (100%)/Ficellomycin (3%)/4 N/A
T1PKS	SGR PTMs (100%)/Hedamycin (87%)/Lasalocid (40%)/Akaeolide (24%)/Lagunapyrone A (22%)/Meridamycin (13%)/A-47934 (8%)/LL-D49194a1 (5%)/2 N/A
Terpenes	Metilisoborneol (100%)/Geosmine (100%)/Isorenieratene (85%)/Hopene (76%)/Hopene (69%)/Cadaside A/cadaside B (9%)/Mediomycin A. (6%)/3 N/A
NRPS	SGR PTMs (100%)/Coelichelin (72%)/Coelibactin (45%)/Streptobactin (35%), (29%)/Rimosamide (21%)/Collismycin A (14%)/Gobichelin A—Gibichelin B (11%)/Ulleungmycin (11%)/Pepticinnamin E (10%)/Cadaside A—Cadaside B (9%)/A-47934 (8%)/Enduracidin (8%)/LL-D49194a1 (5%)/10 N/A

dynamics in insects, our results indicate environmental conditions of the ecosystems may drive the pattern of Actinobacteria taxa obtained.

The association between microorganisms and insects is widespread in nature. Actinobacteria are present in half of the pathogen defense interactions that insects maintain with microorganisms (Kaltenpoth 2009, Seipke et al. 2011), as we mentioned before, fungus-growing ants (Tribe Attini) establish relationships with *Pseudonocardia* (Pseudonocardiaceae) (Cafaro and Currie 2005, Poulsen et al. 2010) to prevent infections in the fungal gardens they cultivate. The protection offered by Actinobacteria against the *Escovopsis* pathogen is so effective (Poulsen et al. 2011) that ants evolve specialized exocrine glands to host them (Poulsen et al. 2003, Currie et al. 2006), among other possible functions (Li et al. 2020). In our study, Actinobacteria belonging to Pseudonocardiaceae (*Amycolaptosis* and *Saccharopolyspora*) were also found in adults of Epiponini. It is possible that Epiponini wasps, like ants, use some microorganisms for protection against pathogens since most of the isolated strains of *Amycolaptosis* and *Saccharopolyspora* showed bacterial growth inhibition *in vitro*.

Microbacterium and *Tsukamurella* strains could be exclusively associated with adults since they have not been found in brood cells

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or immatures (Matarrita-Carranza et al. 2017) to date. There are a few reports of *Tsukamurella* associated with insects (Steinhaus 1941, Hernández and Cafaro 2015), it has been found mostly in soil and clinical material, and some species produce skin infections, meningitis, and pulmonary diseases in humans (Goodfellow and Maldonado 2006). This is the first study that reports *Tsukamurella* strains present in social wasps, and *Microbacterium* in Epiponini wasps. *Microbacterium* has been found in different habitats: soil, plants, insects, dairy products, food, and others (Evtushenko and Takeuchi 2006). From insects, it has been obtained mainly from the gut (Vilanova et al. 2012, Shil et al. 2014, Manfredi et al. 2015, Grigorescu et al. 2018), where its role has been attributed to the production of several enzymes. All strains found in this study were obtained from adult salivary glands. In addition, we found those strains presented inhibition against *B. thuringensis*, *E. coli*, and *P. aeruginosa*. Based on this result we suggest that *Microbacterium* and *Tsukamurella* could provide protection against bacteria, which should be further studied to confirm this and other roles of these strains on the social wasps. In the case of *Microbacterium*, they could also be necessary for enzymatic activities in salivary glands as has been found in the gut of other insects (Vilanova et al. 2012, Shil et al. 2014, Manfredi et al. 2015, Grigorescu et al. 2018).

Actinobacteria such as *Streptomyces*, *Kocuria*, and *Nocardiopsis* have been found in both adults and brood cells (Matarrita-Carranza et al. 2017). *Streptomyces* genus encompasses a group of microorganisms of great medical interest because they can produce more than 100 000 antibiotic compounds (Watve 2001 et al. 2001, Seipke et al. 2011). *Streptomyces* strains used in the pharmaceutical industry are mostly obtained from soil samples. However, recent studies have determined these microorganisms are also associated with invertebrates such as sponges (Taylor et al. 2007, Kamke et al. 2010) and insects (Kaltenpoth et al. 2006, Scott et al. 2008, Haeder et al. 2009, Barke et al. 2010, Poulsen et al. 2011, Seipke et al. 2011, Cambronero-Heinrichs et al. 2019, Chevrette et al. 2019, Matarrita-Carranza et al. 2021, Pessotti et al. 2021, Van Moll et al. 2021). Seven *Streptomyces* strains were isolated from wasp adults in this study. *Streptomyces* may be associated with Epiponini workers for protection as in other insects because these strains also inhibited the growth of several tested bacteria. Nevertheless, *Streptomyces* strains on Neotropical wasps could also be casually obtained from the environment since wasps use plants and soil for nest construction (Wenzel 1998), and *Streptomyces* is found in a wide range of habitats (Seipke et al. 2011).

Kocuria has been reported in the moth *Kunugia latipennis* and is associated with the degradation of phenolic compounds in plant material, as larvae feed on pine leaves (Paul et al. 2012). To our knowledge, this is the first report of the presence of *Kocuria* in Epiponini wasps. *Kocuria* could play a role in workers salivary glands, since all strains were obtained from this structure. In addition, *Nocardiopsis* has been isolated from one species of beetle (Santamaría et al. 2020) and *Apis* (Pronuam et al. 2009, Patil et al. 2010). Moreover, *Nocardiopsis* strains isolated by Santamaría et al. (2020) from the intestine and feces of *Cerambyx welensii* inhibited the pathogen *Micrococcus luteus* due to the production of antimicrobial compounds such as Picromycin, Valinomycin, and Hihydropicromycin. Hence, *Nocardiopsis* isolates in our study could play a role on the external surface of Epiponini workers, since all strains were obtained from the cuticle.

Social wasps' nests are vulnerable to parasite attack due to the accumulation of meconia on brood cells (Jeanne 1991). For this reason, Actinobacteria could be essential for colony survival by protecting against pathogens. This strategy has been used by other solitary wasp species such as *P. triangulum* to protect their larvae (Kaltenpoth et al. 2005, 2006, Kroiss et al. 2010). Epiponini workers immediately insert their head and thorax for ~1 hour, after the fully developed individual leaves the brood cell (e.g. *P. Triangulum*) (Nascimento et al. 2004, Chavarría and West-Eberhard 2010). Given that cells are reused for future larvae, it is possible that Epiponini workers used salivary gland secretions or rubbed their cuticles against chamber walls, in order to sterilize the brood cells. According to Turillazi et al. (2023), gland secretions and microorganisms associated with wasps could produce antimicrobial substances. In addition, through antennation behaviors and trophallaxis (Nascimento et al. 2004, Chavarría and West-Eberhard 2010), adults could transmit Actinobacteria to each other, increasing protection in the colony, as observed by Ishay et al. (2003) in *Vespa orientalis*, where bacteria were transmitted between individuals by trophallaxis. Nevertheless, more studies should be carried out to understand the role of Actinobacteria in Epiponini wasps' cuticle and salivary glands.

Genetic diversity of wasp-associated Actinobacteria with antibacterial potential

Genome analysis confirms the taxonomic identity of six wasp-associated strains, which inhibited the growth of multiple bacteria. Based on the phylogenetic analysis and previous work (Gutiérrez-Araya et al. 2022), strains 6M, 6V, 6T, and 7B might be new species of the genus *Saccharopolyspora*. However, there is a shared ancestor between 6M-6V and 6T-7B, suggesting that they could be genetic variants of the same species. *S. glorisosae* was the closest relative to these strains since they share an ancestor according to the topology of the phylogenetic tree. *S. glorisosae* was originally isolated as a plant endophyte isolated from a plant in the tropical region of Yunnan province, China, and has been recognized for its medicinal properties (Qin et al. 2010).

Furthermore, we confirmed that *Streptomyces* strains 7G and 8L are distant species, which could be associated with their evolution and colonization to different environmental conditions. Strain 7G was collected in La Selva Biological Station, while 8L was collected in Golfito. Climatic parameters are similar in both regions, with high precipitation levels, temperatures varying between 25ºC and 33ºC, and relative humidity ranging between 75% and 80% (IMN 2021, Berger and Schinnerl 2019). Nonetheless, the microenvironment in each ecosystem might drive a different bacterial diversity. These strains were isolated from different parts of the insect, 7G from the salivary glands and 8L from the cuticle. Bacteria isolated from an insect's internal tissue might differ from cuticle bacteria due to the microbial selection occurring in environments with different pressures (Choudoir et al. 2018). For example, for *Protopolybia* sp. it is possible that antimicrobial compounds are produced in the salivary system by symbiotic bacteria, as known for other social wasps such as *P. dominulus* (Turillazzi et al. 2004).

Our results confirmed that strain 7G belongs to *S. globisporus* species. This strain shares a node with *Streptomyces sp*. strain SS07 and *Streptomyces* sp. JS01, suggesting that they are closely related strains of *S. globisporus* species. Similar to 7G, strain JS01 showed antibiotic properties, exhibiting algaecide activity after isolation from the coastal surface waters of the Xiamen Sea (Zhang et al. 2015). On the other hand, SS07 was isolated from the beetle *Monochamus alternatus*, whose natural enemies include fungi such as *Aspergillus fluvus*, *Fusarium oxysporum*, *Bauveria bassiana*, and bacteria such as *Serratia marcescens* (CABI 2022). Therefore, the symbiotic interactions of some *Streptomyces* strains with wasps or other insects could be essential for their survival (Kaltenpoth et al.

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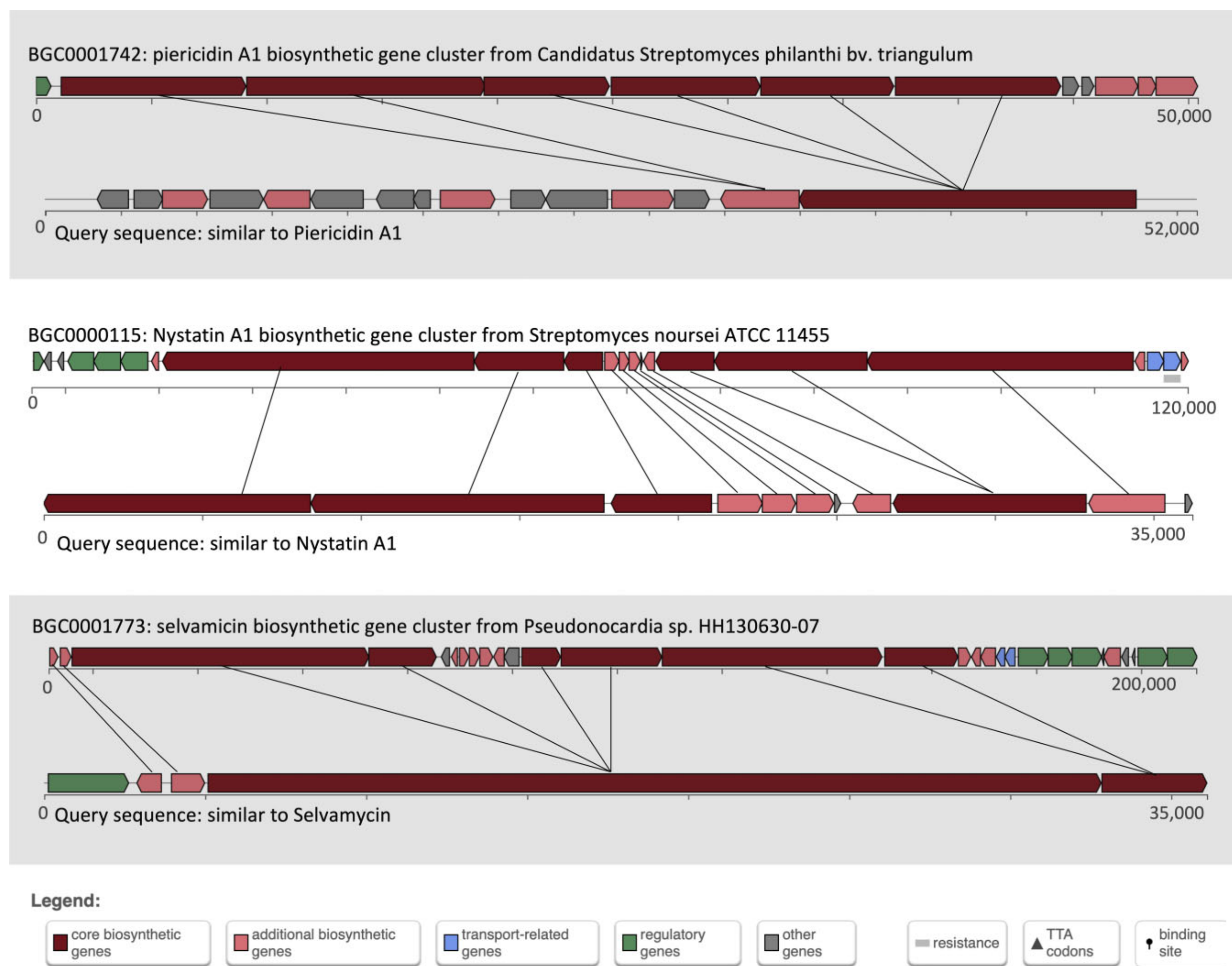

Figure 5. Similarity gene plots between known antimicrobial related BGCs (Piericidin A1, Nystatin A1, and Selvamycin) and predicted BGCs from *Saccharopolyspora* sp. strains isolated from Neotropical social wasp. Genes with different functions are represented by colored boxes, where their function is associated to the color as indicated in the legend. Known clusters were obtained from MIBiG database and similar genes in the predicted BGCs are linked by solid black lines. Predicted BGCs and its genetic similarity were identified with AntiSMASH.

2006, Matarrita et al. 2017). Then, it would be necessary for future studies to confirm whether the observed antimicrobial capacity of *Streptomyces* 7G is related to a potential symbiotic relationship with social wasps.

In our research, we have predicted BGCs that exhibit similarities to known BGCs involved in the synthesis of antimicrobial compounds. Notably, our strains share genetic elements akin to those responsible for producing antimicrobial molecules such as Piericidin A1, Selvamycin, Nystatin, Cinamycin, and Hedamycin. Although our strains' BGCs are not identical to those for the biosynthesis of such molecules, and they might have other biosynthetic routes, they also contain most of the core biosynthetic enzymes responsible for the production of such metabolites (Fig. 5), suggesting that related molecules might be synthesized by the predicted BGCs. Piericidin A1 is a molecule with bioactivity against *Bacillus subtilis*, *Paenibacillus larvae*, *Aspergillus fumigatus*, *A. flavus*, *Penicillium notatum*, *P. avellaneum*, and *Metarhizium anisopliae* (Kroiss et al. 2010). This is the first report of a BGC related to its synthesis in *Saccharopolyspora* sp., which has previously been found in *Streptomyces* from brood cells and antennal glands of *Philanthus triangulum* (Kroiss et al. 2010) and exoskeleton from attine ants (Ortega et al. 2019). Selvamycin is an antifungal molecule discovered in *Apterostigma* ant bacteria, whose primary function has been attributed to the protection of fungal garden nests against fungal pathogens (Van Arnam et al. 2016, Adnani et al. 2017). Nystatin A1 is an antifungal molecule that permeabilizes membranes and can lead to cell death in the presence of another microorganism (Bruheim et al. 2004). This compound has been found in the nest of leaf-cutter ants (Choudoir et al. 2018). Several of the genetic similarities for the biosynthesis of antibiotic compounds we found here, were previously associated with insect-derived microorganisms. Studies have isolated Aentigeromysin, Acyloprolactan, Candidicin D, and Selvamycin, from *Acromyrmex* (Haeder et al. 2009, Barke et al. 2010, Poulsen et al. 2011) and *Apterostigma* (Van Arnam et al. 2016). Additionally, Aycagimycin has been isolated from the beetle *Dendroctonus frontalis* (Oh et al. 2009), and Atreptochlorin and Aiericidin from the solitary wasps *Philanthus* (Kroiss et al. 2010). While our results do not confirm the active production of these specific antimicrobial molecules by our isolates, it evidences the potential of the predicted biosynthetic genes to produce related molecules. Moreover, the alignment of these findings with previous reports, combined with the observed *in vitro* antibacterial activity of these Actinobacteria strains, suggests that they may possess genetic components associated with the synthesis of antimicrobial molecules with a defensive role in the workers of Neotropical social wasps.

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In addition, in most of our strains, a large proportion of BGCs were found with low or no match to known secondary metabolites. Hence, they represent a promising source for exploring natural products. This might be related to the fact that most strains were genetically distant from known species. In this work, *S. globisporus* (7G strain) was the only one classified at the species level, while five out of the six selected strains appear to be new species. Furthermore, the untapped taxonomic variety of bacteria found on epiponine species could be due to specialized functions that Actinobacteria have evolved to survive in the unique environment of social wasps. An arsenal of unknown metabolites is expected to fulfill specific functions in the insect environment, which should be further studied.

Our results present the first report of several Actinobacteria (*Streptomyces*, *Saccharopolyspora*, *Microbacterium*, *Tsukamurella*, *Kocuria*, *Amycolaptosis*, and *Nocardiopsis*) isolated from Epiponini adult wasps with *in vitro* confirmed antibacterial activity. Indeed, in a previous study (Matarrita-Carranza et al. 2017) antibiotic inhibition was tested but only against insect pathogens, and later Matarrita-Carranza et al. (2021) tested the inhibition against different pathogens but only from *Streptomyces* sp. strains isolated from brood cells, not from adults. We have demonstrated here that Actinobacteria associated with social wasp adults have antimicrobial activity and BGCs sharing some genes related to the production of antimicrobial compounds previously described in insects, such as Selvamycin, Piericidin A1, and Nystatin. Most of the strains reported here also showed genomic evidence to presume their classification of novel species, harboring multiple predicted BGCs without any similarities to known BGCs. For these reasons, we speculate that Actinobacteria associated with social wasps could be a potential source of novel antibiotic compounds. Antibiotics from natural sources could be more effective than synthetic ones (Bode 2011), which could help fight the antibiotic resistance crisis. To our knowledge, this is the first research that evaluates Epiponine-associated Actinobacteria and their inhibitory properties to different pathogens.

Author contributions

Laura Chavarría-Pizarro, Kattia Núñez-Montero, Mariela Gutiérrez-Araya, William Watson-Guido, William Rivera-Méndez, and Javier Pizarro-Cerdá.

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