INHIBITORY ACTIVITY OF THERMO-RESISTANT METABOLITES PRODUCED BY Streptomyces SP. CACIS-1.16CA AGAINST HUMAN AND PLANT PATHOGENIC BACTERIA

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ABSTRACT

For many decades, members in the genus Streptomyces have been considered the principal microbial producers of secondary metabolites with antibiotic activity. In this study, we evaluated the inhibitory activity of a cell-free supernatant produced by Streptomyces sp. CACIS-1.16CA against human and plant bacterial pathogens. We also assessed the thermal stability of the compounds. An initial screening to evaluate the antibacterial activity of compounds diffused from a CACIS-1.16CA culture into agar discs found inhibitory effects against Escherichia coli, Staphylococcus aureus, Enterococcus faecalis, Bacillus pumilus, and Pectobacterium carotovorum. A cell-free supernatant from cultured CACIS-1.16CA confirmed the inhibition effects on the susceptible pathogens. Thermally treating the supernatant did not significantly affect the inhibitory activity, indicating Streptomyces sp. CACIS-1.16CA produces some thermally stable antibacterial metabolites. This antibacterial activity is consistent with the detection of biosynthetic gene clusters for polyketide synthase Types I and II and non-ribosomal peptide synthase.

Keywords: antibiotic; pathogenic bacteria, Pectobacterium carotovorum, secondary metabolites, streptomyces

INTRODUCTION

Streptomyces (Streptomycetaceae) are the group of microorganisms considered to have the highest production of bioactive compounds, and for many years they have been considered the best source of new antibiotics (Genilloud, 2017). The genus Streptomyces belongs to this bacterial family, and with more than 600 diverse species described, they produce more than half of the known antibiotics (Nithya et al. 2018). Other biologically active compounds produced by members of the genus include metabolites that function as plant growth factors, siderophores, anti-protozoans, antifungals, antivirals, insecticides, pesticides, herbicides, immunomodulators, antitumor agents, and enzyme inhibitors (Harir et al. 2018). Despite this diversity of compounds, the discovery of new antibiotics has significantly declined, which is why new strategies have been developed for finding novel molecules. One of these strategies relies on the isolation of microorganisms in unexplored environments, with the goal of identifying new bioactive compounds. Microorganisms inhabiting oceans, soil halophiles, plant endophytes, and less studied species have been preferred. For example, a genomic study of Streptomyces yeochonensis CN732, an acidophilic neutrotolerant bacterium isolated from soil, identified 22 biosynthetic clusters of secondary metabolites. This antibacterial activity is consistent with the detection of biosynthetic gene clusters for polyketide synthase Types I and II and non-ribosomal peptide synthase.

Streptomyces sp. CACIS-1.16CA is a soil bacterium isolated from a Wetland of International Importance (Ramsar site) located at Los Petenes Biosphere Reserve in Campeche, State, Mexico. This bacterium has shown antagonistic activity against the phytopathogenic fungi Curvularia sp., Aspergillus niger, Helminthosporium sp., Fusarium sp., Alternaria sp., Phytophthora capsici, Colletotrichum sp., and Rhizoctonia sp., all isolated from diseased plants. The CACIS-1.16CA strain produces a yellow water-soluble metabolite that accumulates in culture medium and correlates with an inhibitory effect on fungal growth (Evangelista-Martinez, 2014). Streptomyces are also important for producing secondary metabolites with anti-candida activity (Córdova-Dávalos et al. 2018). In view of the above, the objectives of this study were to evaluate the antibacterial activity of a cell-free supernatant produced by Streptomyces sp. CACIS-1.16CA against human and plant pathogens and to determine the thermal stability of the compounds in vitro.

MATERIAL AND METHODS

Microorganisms and cultures

Human pathogens used in this study were Escherichia coli ATCC 17775, Salmonella enterica subs. enterica serovar Typhimurium ATCC 14028 (Salmonella Typhimurium), Staphylococcus aureus ATCC 25923, Enterococcus faecalis ATCC 29212, Salmonella enterica serovar Enteriditis ATCC 33090 (Salmonella Enteriditis), and Pseudomonas aeruginosa ATCC 33090. The plant pathogen bacteria were Pseudomonas syringae pv. phaseolicola, Bacillus pumilus, and Pectobacterium carotovorum. All strains were maintained on nutrient agar (NA), excepting the Pseudomonas species, which were grown on King B agar. The bacterial inoculums were prepared in Mueller-Hinton (MH) broth and King B broth, respectively. The Streptomyces sp. CACIS-1.16CA strain was maintained on International Streptomyces Project No. 2 Agar (ISP2).

Preparation of bacterial cultures

Bacteria inoculums were prepared according to the Clinical and Laboratory Standards Institute method (CLSI, 2012). The broth media was inoculated with a loopful of a bacterial colony, mixed, and maintained in an orbital shaker (150 rpm, 18 h at 37 °C). A 0.1 mL volume of a bacterial suspension was adjusted to a 0.5 optical density (OD₅₆₀) on the McFarland scale and dispersed on MH or King B agar media in a Petri dish. The Petri plates were ready to test for antibacterial activity after absorption of the cell suspension.
Evaluation of antibacterial activity

A preliminary screening to evaluate the inhibitory effect of diffusible metabolites was performed using the agar disc-diffusion method. Initially, a suspension of CACIS-1.16CA spores was distributed on NA medium at the edge of a Petri dish, leaving a square space without bacteria (3 cm²) at the center. The inoculated plates were incubated for 14 days at 29 °C. The growth of the strains was then inactivated by placing the Petri plates in a sealed chamber containing chloroform for 30 min. Subsequently, a 6 mm diameter mycelial-free agar disc was removed from the center of the Petri dish, deposited on seeded plates inoculated with pathogenic bacteria (as described above), and incubated at 37 °C for 24 h. The diameters of the inhibition halos were measured with a caliper, with all measurements conducted in triplicate.

Production of antibacterial metabolites in submerged cultures

ISP2 medium supplemented with 50 ppm of antifungal A (Sigma-Aldrich) was used. Cultures (30 mL) were grown at 30 °C in 250-mL siliconized flasks with stainless steel springs at 150 rpm in an orbital shaker (Kieser et al. 2000). Cultures were inoculated with a spore suspension that had been washed twice in sterile distilled water and adjusted to an initial optical density of 0.05. Growth was monitored by optical density measurements at 450 nm. Samples to be assayed for antibacterial activity were drawn at appropriate time intervals and centrifuged at 16,000 × g in a microcentrifuge (Hettich); the supernatants (SN) were then withdrawn, and filter sterilized (0.22 μm, Millipore) into fresh tubes before storing at −20 °C until the assays. The SNs were analyzed using a Jenway 6715 UV/VIS spectrophotometer (Cole-Palmer), with the absorbance spectra ranging from 220 to 740 nm. All measurements were conducted in triplicate.

Evaluation of SN antibacterial activity by the paper disc-diffusion assay

The production of inhibitory compounds accumulated during the growth of the CACIS-1.16CA strain was analyzed by the paper disc-diffusion method (Carovati-Stanko et al. 2010). These experiments were performed using the pathogenic bacteria that were inhibited in the previous tests. A 10 μL SN fraction collected after 96 h of incubation was deposited on 6 mm diameter filter paper discs (Whatman) and placed on the pathogen-inoculated Petri plates. After 24 h of incubation at 37 °C, the diameters of the inhibition’s zones were measured with a caliper. Kanamycin (30 mg/mL) was used as the positive control. All experiments were conducted in triplicate.

Effect of temperature on antibacterial activity

At this stage of the study, the effect of temperature on the antibacterial activity of the compounds produced by Streptomyces sp. CACIS-1.16CA was evaluated. Initially, the total SN fractions collected from the submerged cultures were heated to 100 °C for 3 min and assayed on E. coli ATCC 17775. Subsequently, one 96 h SN fraction was heated at 100 °C for 60 min, and another SN fraction was treated at 121 °C for 15 min in an autoclave. These two treatments were evaluated on the five selected pathogenic strains. The determination of antibacterial activity of the SNs was performed as described previously, with 10 μL of the SN deposited on 6 mm diameter filter paper discs before placement on the surface of MIH Petri dishes inoculated with the pathogenic bacteria. In all cases, the Petri plates were incubated at 37 °C for 24 h, and all experiments were performed in triplicate. The diameter of the inhibition zone was measured with a caliper. For the last experiment, the residual activity (RA) of the SNs was determined based on Carvalho and Van Der Sand (2010), where RA (%) = (MSNT – 6) / (MSNC – 6) × 100. MSNT is the mean size of the inhibition halos obtained with the heat-treated SN (mm); MSNC is the mean size of the inhibition halo (mm) for the heated control sample; and 6 is the diameter of the paper disc (mm).

Detection of polyketide synthase Type I and Type II genes (PKS Type I, PKS Type II) and non-ribosomal peptide synthase genes (NRPS)

Detecting the biosynthetic genes involved in the production of molecules with antimicrobial activity in Streptomyces sp. CACIS-1.16CA was performed by polymerase chain reactions (PCR) with specific oligonucleotides. PKS Type I fragments were amplified with the degenerate oligonucleotides KIF (5’- TAAAGTSSACAAATCTGCAGCBA-3’) and M6R (5’- CGCAAGGTCSGCTTGACAGTA-3’). The NRPS gene fragments were amplified with the oligonucleotides A3F (5’- GCATCCTATATACCTGCAGC-3’) and A7R (5’- ASAGTCCVCCGCTGATAS-3’, Ayuso-Sacido and Genilloud, 2005), and the Type II gene fragments were amplified with the degenerate oligonucleotides KSo (5’- TSGSCTGCTCTACGATC-3’) and KSB (5’- TGGAAANCCGGCGAABCCGCTC-3’) (Metsä-Ketelä et al. 1999). The PCR conditions were implemented using Expand High Fidelity PCR system (Roche) (Ayuso-Sacido and Genilloud, 2005; Metsä-Ketelä et al. 1999). The PCR products were visualized in 1.5 % agarose gel prepared in 1× Tris-Borate buffer (TBE; Invitrogen) and stained with ethidium bromide.

Data analysis

The inhibition halo sizes from the diffusion experiments are expressed as means ± standard deviation (SD). The means were compared using an analysis of variance (ANOVA) followed by the Fisher test (p = 0.05). A statistical analysis to evaluate the effect of temperature on the antibacterial activity of the SNs was performed with an ANOVA followed by the Dunnet test (p = 0.05). The statistical analyses were performed with the MiniTab v18 program (MiniTab, LLC).

RESULTS

Antibacterial activity of Streptomyces sp. CACIS-1.16CA

The initial screening to evaluate the antibacterial activity of Streptomyces sp. CACIS-1.16CA had the purpose of selecting those human and plant pathogenic bacteria susceptible to the compounds produced by the strain. The microorganisms inhibited by the compounds that spread to the agar discs were E. coli, S. aureus, E. faecalis, B. pumilus, and P. carotovorum (Table 1). Significant differences were found among the susceptible bacteria, with E. coli and B. pumilus having the antibacterial activity halos and S. aureus having the smallest. These results indicate that the extracellular metabolites produced by the CACIS-1.16CA strain diffused into the growth medium and exerted an inhibitory activity against the bacteria.

Table 1 Primary screening to detect bacteria inhibition by Streptomyces sp CACIS-1.16CA

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Inhibition halo (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus</td>
<td>12.7 ± 0.8</td>
</tr>
<tr>
<td>Salmonella Enteriditis</td>
<td>-</td>
</tr>
<tr>
<td>Salmonella Tiphymurium</td>
<td>-</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>13.8 ± 0.4</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>13.4 ± 0.5</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>-</td>
</tr>
<tr>
<td>Pseudomonas syringae pv syringae</td>
<td>-</td>
</tr>
<tr>
<td>Bacillus pumilus</td>
<td>13.7 ± 0.2</td>
</tr>
<tr>
<td>Pectobacterium carotovorum</td>
<td>13.0 ± 0.4</td>
</tr>
</tbody>
</table>

Media non-shared letter are significantly different (p < 0.05). (-) No inhibition detected.

Growth phase-dependent production of antibacterial compounds

The production of antibacterial metabolites was examined to determine the growth phase in which the production of the inhibitory metabolites begins. A growth curve for Streptomyces sp CACIS-1.16CA was determined for 0 to 156 h and antibacterial activity of supernatants on E. coli was evaluated. Assays using the filter paper disc-diffusion method detect inhibitory halos with SN’s starting at 48 h, which coincides with the exponential growth phase. The extracellular compounds accumulated during the stationary growth phase reaching the maximum inhibitory activity at 96 h (Figure 1). Bacteria from the Streptomyces genus are characterized by their production of extracellular metabolites with antibiotic activity and a battery of hydrolytic extracellular enzymes, which can act synergistically to inhibit the growth of microorganisms (Chater et al., 2010). Based on this, a heat treatment of 100 °C for 3 min (a) was applied to the SNs from the different culture times to confirm whether the antibacterial activity was due solely to secondary metabolites, or if hydrolytic components contributed to the inhibition. The heat treatments did not affect the SNs ability to inhibit E. coli, resulting in an inhibition halo similar to that of the unheated sample (Figure 1). Therefore, there were no statistically significant differences (p > 0.05, Dunnet test) between the heated and unheated SNs. In general, a direct correlation between the diameters of the inhibitory halos and the growth phases of the CACIS-1.16CA strain was observed.

Figure 1 Growth of Streptomyces sp. CACIS-1.16CA and the antibacterial activity of the supernatant against Escherichia coli. An aliquot of SN was heated to 100 °C for 3 min (a); unheated control SN ( ). O.D., optical density. No significant differences (p > 0.05, Dunnet test). The inner box showed the antibacterial activity for 96 h SN
The ultraviolet/visible (UV/Vis) absorption spectrum of the collected SNs confirm the accumulation of extracellular metabolites that could exerted the inhibitory activity (Figure 2). The spectral scan detected two maximum absorption peaks. Peak 1 is between 380 and 400 nm (at the UV spectrum limit), appears at 60 h of growth, and continues to accumulate until 156 h. Peak 2 is between 420 and 440 nm (visible spectrum) and begins at 60 h of growth, though in a smaller proportion than Peak 1, except at 156 h, when maximum production is reached.

An evaluation using the filter paper disc-diffusion method using 96-h SN confirmed that the extracellular compounds accumulated during the growth of the Streptomyces sp. CACIS-1.16CA strain have antibacterial activity against other pathogenic bacteria (Figure 3). There were significant differences between the means, indicating that E. coli and E. faecalis were the most susceptible to inhibition, while S. aureus, with the smallest inhibitory halo, was the least susceptible.

**Thermal stability of the compounds produced by Streptomyces sp. CACIS-1.16CA**

Considering the thermal stability of compounds contained into the supernatants, the 96-h SN was exposed to the more drastic conditions of 100 °C for 60 min and 121 °C for 15 min (Figure 4). These two treatments were evaluated with the five pathogenic bacteria using the paper disc-diffusion method. The RA of the SN determines the percentage of activity retained by the heat-treated SN in comparison to the unheated SN. The ANOVA results and Dunnet test (p > 0.05) indicated there were no significant differences between the SNs heated to 100 °C for 60 min and the control. Under these conditions, the treated SN retained its antibacterial activity above 94 %. Statistically significant differences were observed between the SN treated at 121 °C for 15 minutes respect to the unheated control SN and the SN heated for 60 min at 100 °C (ANOVA and Dunnet test, p < 0.05). Nevertheless, despite being exposed to drastic conditions (autoclaved), the SN retained more than 80 % of its antibacterial activity against the pathogenic bacteria (Figure 4a). This can be seen in Figure 4b, which shows the inhibitory halos diminished by between 10 and 20 %. The decreased activity may be due to the destruction of other antibacterial compounds present in the mixture.

**Detection of biosynthetic gene clusters of secondary metabolites**

The biosynthetic genes in the PKS-I, PKS-II, and NRPS clusters in the Streptomyces sp. CACIS-1.16CA genome were detected by PCR (Figure 5). The amplified fragments corresponded to ~700 bp for NRPS, to ~1300 bp for PKS-I, and to ~ 900 bp for PKS-II. These results indicate a potential for the strain as a producer of compounds with antimicrobial activity and a different chemical nature.
DISCUSSION

Bacterial resistance to antibiotics is a wide-spread problem, with the most important consequence being a growing number of compounds that are no longer effective in treating disease (Lin et al. 2015). This has led to the search for new strategies to combat the emergence of new compounds (Geniloud, 2017; Hug et al. 2018). New compounds can be discovered by screening new natural products or by synthesizing new compounds (Ren et al. 2019). The form of new compounds by using strategies that allow for the quick evaluation of a large number of molecules (Murray et al. 2019). However, a great number of studies are still based on traditional microbiological methods and focus on screening extreme and little-explored environments to find novel microorganisms producing new antibacterial compounds (Geniloud, 2017; Hug et al. 2018). The presence of new compounds is often determined by changes in their concentration, which depends on the number of double bonds in the chromophore (Barreales and Aparicio, 2021).

Bioinformatic gene clusters are organized groups of genes involved in the production of specialized metabolites, some of which display antimicrobial activity. Polyketide synthase genes (PKS Types I and II) as well as genes from non-ribosomal peptide synthetases (NRPS) are detected in Streptomyces sp. CACIS 1.16CA, which indicates a high possibility to macrocyclic polyketides (PKS-I), polycyclic aromatic polyketides (PKS-II), and non-ribosomal peptide compounds (NRPS). Streptomyces are a widely recognized group of antibiotic producers, with genomes containing more than 20 biosynthetic gene clusters for secondary metabolites. For example, the genome of S. avermitilis contains 5% of its genome (Challis and Hopwood, 2003). Whole genome sequencing and analysis of Streptomyces fildesensis showed a 9.47 Mb genome size with 42 predicted biosynthetic gene clusters and 56 putative clusters representing a 22% of total genome content (Nuñez-Montero et al. 2019). It is important to point out that, in addition to the fact that many Streptomyces species have PKS Types I and II and NRPS bioinformatic clusters, some species also produce a third type of synthase. PKS Type III has the peculiarity that it does not use an acyl-carrying protein to produce metabolites (Risdian et al. 2019).

CONCLUSION

With the emergence and rapid spread of resistant bacteria in a diversity of fields, the world needs new antibiotics to be developed, and not only for treating human diseases. We need new antimicrobial molecules to control the emergence of food-borne pathogens, plant bacterial pathogens, and nosocomial infections. We also need drugs that can stop bacteria by preventing their ability to form biofilms or by affecting their chemical communications with each other. The isolation of microorganisms from unexplored environments with a goal of identifying new bioactive compounds is an important strategy for finding novel antibiotics. Our experimental findings describe the production of heat-resistant compounds produced by a Streptomyces species isolated from a poorly studied ecosystem. Future work on these compounds should focus on separating and purifying the compounds to probe their activities against drug-resistant bacteria or superbugs. The isolation of these compounds from the crude extracts and chemical analyses would be necessary for structure elucidation.

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REFERENCES

Tunicamycin, a nucleoside antibiotic (870 Da) identified from culture broth of Streptomyces lysosuperficis showed an absorption spectrum with a maximum absorption peak at 260 (Atta, 2011). Generally, polyene quantification is based on their characteristic UV-visible absorption spectrum, in aqueous or organic solvents and is the basis for the detection of many antibiotics which depends on the number of double bonds in the chromophore (Barreales and Aparicio, 2021).

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