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ORIGINAL PAPER

Involvement of cyclodipeptides in the competition of bacterial communities in the oligotrophic Churince aquatic system of Cuatro Ciénegas Basin dominated by Gammaproteobacteria

Enrique Martínez-Carranza[1,2] · Gabriel Y. Ponce-Soto[3] · Alma L. Díaz-Pérez[1] · Erasmo Cadenas[4] · Valeria Souza[3] · Jesús Campos-García[1]

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Abstract The Cuatro Ciénegas Basin (CCB) within the Chihuahuan Desert in México is an extremely oligotrophic oasis with negligible phosphorous levels, described as a hot spot of biodiversity, not only in stromatolites and microbial mats, but also in living forms in general. The microorganisms possess the capability to produce a wide variety of virulence factors, antibiotics, and quorum-sensing (QS) crosstalk signals such as non-ribosomal cyclodipeptides (CDPs) which enables them to colonize diverse ecological niches. In the aquatic system of CCB known as Churince, a bacterial population was isolated from the Lagunita pond dominated by Gammaproteobacteria. In this work, we determined the relationships between the antagonism and CDPs production in this bacterial population. Results indicate that 68% of isolates showed antagonistic effects over other isolates, correlating with production of CDPs and the antibiotic 2,4-diacetylphloroglucinol (DAPG). Although a minority of the isolates were capable of inducing a QS biosensor strain, bacterial QS interference was not the main mechanism in the antagonism observed. Thus, our results indicate that CDPs primarily, and DAPG to a lesser degree, are involved with the growth-inhibition competition mechanisms of bacterial communities in the Lagunita pond and was associated with a Gammaproteobacteria dominancy phenomena.

Keywords Oligotrophy · *Pseudomonas* · Cyclodipeptides · Quorum sensing · Bacterial population control

Introduction

The Cuatro Ciénegas Basin (CCB) is an oasis of about 840 km^2 in the shape of a butterfly bordered by high sierras, which has been described as a hot spot of biodiversity within the Chihuahuan Desert in México (Souza et al. 2006, 2012). An important characteristic of the aquatic habitats in this area is that they are extremely oligotrophic due to the almost negligible phosphorous levels (Escalante et al. 2008; Ponce-Soto et al. 2015; Souza et al. 2008). In the last 10 years, several works unfolded a very large microbial biodiversity that includes marine relict lineages (Bonilla-Rosso et al. 2012; Escalante et al. 2008; Lopez-Lozano et al. 2012, 2013; Moreno-Letelier et al. 2012; Pajares et al. 2012, 2013; Peimbert et al. 2012; Souza et al. 2006, 2012). Within those studies, selective isolation of *Pseudomonas* and *Aeromonas* bacterial genus showed that population shifts in function to seasonality in a desiccation lagoon of CCB (Rodriguez-Verdugo et al. 2012), documenting that many of the *Pseudomonas* populations release chemical compounds into the environment, thus inhibiting the competitor populations. Nevertheless, in a

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✉ Jesús Campos-García
jcgarcia@umich.mx

[1] Laboratorio de Biotecnología Microbiana, Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo, Edif. B-3, Ciudad Universitaria, 58030 Morelia, Michoacán, Mexico

[2] Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Mexico, Mexico

[3] Instituto de Ecología, Universidad Nacional Autónoma de México, Mexico, Mexico

[4] Facultad de Ingeniería Mecánica, Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán, Mexico

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mesocosm enrichment experiment in the Churince aquatic system of the CCB, it was found that after an increase in P and N, the presence of strains with antibiotic resistance and the capability to form biofilms was reduced as was the abundance of *Pseudomonas* and the increase of other Gammaproteobacteria (Ponce-Soto et al. 2015; Lee et al. 2017). In general, the *Pseudomonas* genus possesses the ability to colonize many environments, such as water, soil, plants, and animals, including some important pathogens (Battle et al. 2008; de Abreu et al. 2014). However, the reason of their decline in Churince lagoon could be more related to their capabilities of interaction by quorum sensing (QS) with their communities to share rare recourses. The QS phenomenon has been also carefully explored (de Kievit and Iglewski 2000; Fuqua and Greenberg 2002; Lee and Zhang 2015). In this context, a novel QS communication system has been recently described, involving the signal molecule called IQS which is synthesized by a non-ribosomal peptide synthase (NRPS) (Dandekar and Greenberg 2013; Lee et al. 2013; Lee and Zhang 2015). The CDPs are cyclic molecules comprised of two amino acid residues bound by peptide bonds; they are produced by a wide range of organisms, from bacteria to fungi and animals (Seguin et al. 2011). In addition, the CDPs derived from NRPS are considered as signal molecules (Rojas Murcia et al. 2015). CDPs belong to the non-ribosomal peptides that are synthesized in diverse organisms by NRPS and CDP synthases, enzymes that utilize free amino acids or aminoacylated transfer RNAs (aa-tRNAs) as substrates (Bonnefond et al. 2011; Miller and Gulick 2016). Thus, CDPs possess a potential role as inter-kingdom signals, however, its mechanism of action, physiology, and ecological relevance are poorly understood (Gonzalez et al. 2017; Holden et al. 1999; Ortiz-Castro et al. 2011).

The production of CDPs in bacterial ecosystems could be considered as an additional mechanism of community identification. Moreover, it can be proposed that CDPs act as competition-signaling molecules, in agreement with the microorganism Kin Competition model and the evolution of cooperation model (Platt and Bever 2009), where the compounds eliminate competition from non-genetically related bacterial populations. This controversy between a cosmopolitan successful genus versus locally susceptible strains led us to create a chemical hypothesis. If *Pseudomonas* and other Gammaproteobacteria species from Churince aquatic system of the CCB are able to colonize the water environment of an extreme ecosystem, then we should find the particular molecules that allow them to survive in a complex and diverse aquatic community. The logical molecular candidates are antibacterial or QS-inhibitor compounds. Herein, we describe the interactions between a bacterial community from the Churince aquatic system of CCB, evaluating the bacterial antagonistic activity correlation with the bioactive compound production.

Materials and methods

Study site, bacterial strains, and culture conditions

In May 2011, a nutrient enrichment experiment was performed in Lagunita (26.84810°N, 102.14160°W), lateral to the main Churince flow system (Lee et al. 2015). This experiment was done using mesocosms under three different N:P regimes (P only, N:P = 16 and NN:P = 75), while the control mesocosms were left unamended. After 3 weeks of fertilization, more than two-thirds of the applied P was immobilized into seston or sediment. To observe the changes in cultivable Gammaproteobacteria, 15 mL sterile falcon tubes were used to sample each mesocosm in triplicate before and after the experiment.

From those tubes, 100 µL were taken from each tube and plated in situ using the selective medium Pseudomonas isolation agar (PIA) and grown at an environmental temperature (20–35 °C) as previously described (Ponce-Soto et al. 2015). Once single colonies were developed, a sample was used for DNA extraction and genotyped using 16 rDNA, while other parts of the sample were frozen in glycerol vials; another part was used for testing antibiotic resistance and biofilm formation (Ponce-Soto et al. 2015). However, not all of them could be genotyped due to the inhibitory response of most of their DNA toward the PCR reaction. Even though PIA is a Pseudomonas-specific medium, the organisms identified by 16S rDNA sequence (62%) represent all of them Gammaproteobacteria. The 61 bacterial isolates that were used in this study are: *Pseudomonas fluorescens* (AP19, 1C9, 1C11, 2C5, and 2C9), *Pseudomonas aeruginosa* (1D4, ENNP23, and ENNP25), *Pseudomonas* sp. (AP42, 2C1, 2C2, 2C8, 2E2, 2E3, 2E4, 3C7, 3C8, 3D8, and 4D1), *Aeromonas veronii* (ANP5, ANNP30, and DNP9), *Aeromonas* sp. (3E2 and 3E3), *Halomonas* sp. (1A5, 1A8, 1A9, and 2A8), *Rheinheimera* sp. (2A3, 2A4, 2A9, and 1B6), and 48% of unidentified isolates (AP10, 1A10, 2B7, BC45, CNNP19, CNNP27, CNNP28, CNNP34, CNP39, CP14, CP18, CP26, CP27, CP28, CP29, CP30, CP31, CP32, CP33, CP34, CP35, CP36, CP37, CP38, CP39, CP40, CP41, CP49, and CP50). Table S1 shows the origin, nutrient input and activity of all the strains (Table S1, supplementary material).

All samples were collected under the permit SGPA/DGVS/00614/13 FAUT–0230 granted to VS by the Secretaría de Medio Ambiente y Recursos Naturales (SEMARNAT, México). Endemic bacterial strains from the same site *Bacillus coahuilensis* (Alcaraz et al. 2008) and *Pseudomonas cuatrocienegasensis* (Escalante et al. 2009), as well as strains from our laboratory collection: *Pseudomonas aeruginosa*

PAO1, *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus* (Diaz-Perez et al. 2004). For the purposes of our study, strains were grown in Luria-Bertani (LB) broth or M9 minimal medium (MM9) with 0.2% (w/v) glucose at 30 °C with shaking. Solidified media was prepared by adding 1.5% (w/v) agar.

In vitro assay for bacterial antagonist interaction

Growth inhibition of bacterial strains was evaluated by in vitro assays to determine the antagonist interactions between the isolates. The strains to test were first grown in LB medium at 30 °C to exponential phase and optical density (OD) between 0.4 and 0.7 at 600 nm; a drop of 10 µL of culture (the sender strain) was inoculated in the center on the surface of an LB agar plate and incubated 4 h to favor its growth. After, a drop of 10 µL of each receiver isolates were placed around the sender strain (antagonist); six strains by plate were tested. The plates were incubated for 24 h at 30 °C, and inhibition halos were measured using a ruler. These assays were carried out by triplicate using the 61 isolates in all-against-all tests (61 strains were tested as sender and also as receivers, rendering 3721 interaction data). Under growth-inhibition assays, we determined the antagonistic activity utilizing a relative scale of growth-inhibition halo (from 0 to 5), where 0 corresponds to null growth-inhibition and 5 value corresponds to the highest growth-inhibition effect (Fig. 1a). The quantitative data were analyzed by an interaction matrix using a five-value scale as shown in Fig. 1.

Solvent extraction and chemical analysis of antimicrobial compounds from bacterial extracts

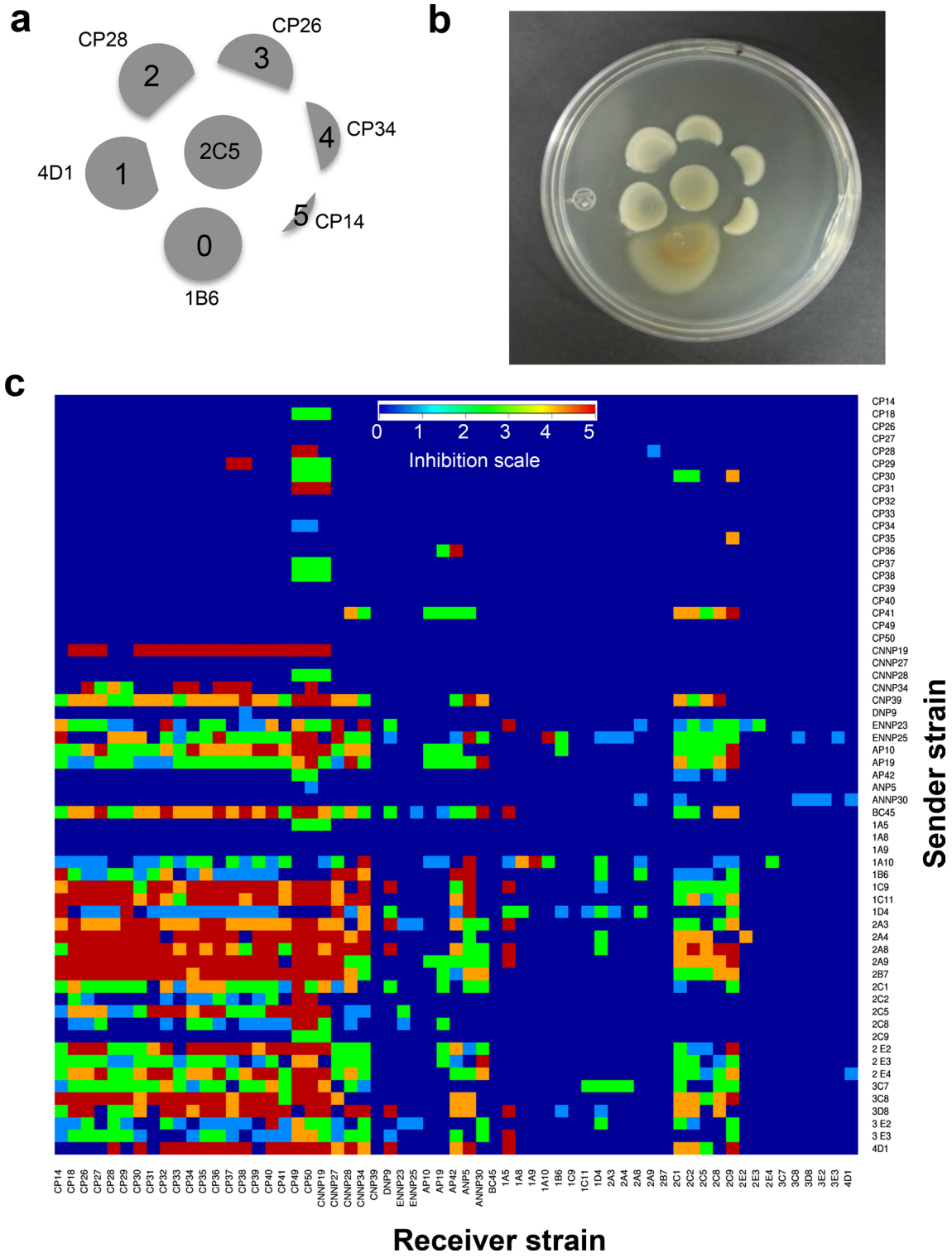
For the antimicrobial compound extraction from bacterial extracts, a 2.5×10^8 CFU inoculum of each bacterial strains isolated from CCB was placed in 100 mL of MM9 medium and incubated in a growth cabinet for 48 h at 30 °C. Cell-free supernatants were prepared by centrifugation (10,000×*g* at room temperature by 10 min). The resulting supernatant was extracted twice with two volumes of ethyl acetate supplied with acetic acid (0.1 mL/L). The extracts were evaporated to dryness using rotavapor at 60 °C with vacuum. The residue was dissolved in methanol:acetonitrile (1:1), concentrated and dissolved to 1 mL in HPLC-grade acetonitrile. Analysis of extracts was carried out using high performance liquid chromatography (HPLC) using a photo diode array detector and a reverse-phase HPLC column Sephasil-Peptide C18, 12 µm, 4.6 mm × 250 mm (Amersham). Samples were eluted with water:acetonitrile, starting with an equilibration solvent mix of 0:100, followed by a linear gradient up to 60:40, at a flow of 1 mL/min for 15 min, following with a return to 0:100 solvent mix in 3 min and an equilibrium phase lasting 2 min. The deionized water and HPLC-grade acetonitrile were filtrated and degasified. The extracts also were analyzed for identification of CDPs by gas chromatography coupled with mass spectrometry (GC–MS), as previously described (Ortiz-Castro et al. 2011). Synthetic CDPs were used for structure confirmation corresponding to cyclo(Pro-Val), cyclo(Pro-Tyr), and cyclo(Phe-Pro) (G-4730, G-4715, and G-4720, respectively, were acquired from Bachem Co. CDPs quantitation was determined as relative area units of the peaks observed in chromatograms from the HPLC or GC–MS analysis utilizing the synthetic CDPs as standard compounds.

Determination of the effect of bacterial extracts over growth inhibition of the isolates

Total solvent-obtained extracts from each of the 61 bacterial isolates were utilized for determination of inhibition of bacterial growth, by a diffusion of compounds assay. Susceptible strain *B. coahuilensis* was first grown in LB medium at 30 °C to exponential phase; 10 µL of the culture was plated on an LB agar plate to have a bacterial lawn. Later, ten filter paper disks (6 mm diameter) were situated over the bacterial lawn, and the extracts from the bacterial cultures were added on the disks at different concentrations. The plates were incubated for 24 h at 30 °C, and inhibition halos on the bacterial lawn were measured. Inhibition assays were carried out in triplicate.

Determination of the effect of bacterial extracts on quorum sensing

The effect of bacterial solvent-obtained extracts on violacein production for the detection of *N*-acylhomoserine lactones was determined utilizing the biosensor mutant strain of *Chromobacterium violaceum* CV026 (McClean et al. 1997). The CV026 strain was grown in LB liquid medium at 30 °C to its exponential phase (OD between 0.4 and 0.7 at 600 nm), 10 µL of the culture of the biosensor strain was plated on top of the LB agar plate to have a bacterial lawn. Ten filter paper disks (6 mm diameter) were situated carefully on top of the LB plate surface (over the biosensor lawn). Extracts proceeding from the bacterial cultures were added on top of the filter paper disks. The violacein induction over the plated strain was tested at different concentrations of extracts. The plates were incubated for 24 h at 30 °C. Afterwards, the response was determined by detection of the violacein halo (purple pigment) produced by the biosensor strain CV026. The assays were carried out in triplicate.



Statistical analysis

For interaction matrix plot, 3721 interaction data obtained of the antagonistic assay between the 61 isolates were analyzed with R software v2.12.1 (The R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0). The datasheet fed with the 3721 interaction data proceeding from antagonistic assay, plus data of bacterial growth inhibition by extracts, and/or with the data of peak area of compound content in bacterial extracts determined by HPLC were analyzed by principal component analysis (PCA) using the STATISTICA software (Data Analysis Software System

◂**Fig. 1** Matrix of antagonistic interactions by the bacterial isolates from Lagunita Pond of the Cuatro Ciénegas Basin. **a** Scale of growth inhibition used for acquisition of data for matrix of antagonistic interaction generation. Sender strain (2C5) is inoculated in the center of the plate and isolates to test are around called as receivers (1B6, 4D1, CP28, CP26, CP34, and CP14). Antagonistic activity was determined utilizing a scale of growth-inhibition halo, where 0 corresponds to null growth inhibition and 5 corresponds to the highest growth inhibition. **b** Growth-inhibition assay for determination of antagonistic effect of the isolates. LB plate was incubated by 24 h at 30 °C. *P. fluorescens* (2C5) as sender strain tested against the receiver strains *Rheinheimera* sp. (1B6), *Pseudomonas* sp. (4D1), the unidentified strains (CP26, CP28, CP34, and CP14). **c** Matrix of antagonistic interactions rendered by the 61 isolates (senders) against their self (receivers) using the data of 3721 resulting interactions as indicated in **b**. The growth-inhibition scale is shown in relative values from 0 (blue) to 5 (red) in the color inhibition scale. Data represent the measurements done in three replicates, and the results are expressed as the arithmetic mean ± SD, analyzed with R software v2.12.1 (The R Foundation for Statistical Computing, Vienna, Austria)

8.0. Stat Soft. Inc.). Additionally, all data values were normalized in a scale of 0–1.0 and analyzed by PCA using the IBM SPSS statistics v22, (https://www.ibm.com/analytics/mx/es/technology/spss/) as first test and after components matrix by *k*-means using the Matlab software (https://www.mathworks.com/help/stats/kmeans.html). Other data were statistically analyzed using GraphPad Prism 6.0 software.

Results

Bacterial antagonism and growth inhibition by extracts of isolates from the Lagunita pond of the Churince aquatic system

The Churince flow system, located at the western region of CCB, is distinguished by gypsum sediments and has a strong longitudinal gradient of salinity, temperature, pH, and dissolved oxygen (Cerritos et al. 2011). This site is characterized by low phosphorus concentrations, often less than 0.1 µM or below detection, and high N:P ratios (> 200:1). From this oligotrophic ecosystem, we analyzed a group of 61 bacterial isolates proceeding from the Lagunita pond of the Churince flow system, of which 33 were identified by 16S rDNA sequencing (Ponce-Soto et al. 2015). We determined their antagonistic activity by measuring the growth-inhibition halos between 61 bacterial isolates (Table S1), where 0 corresponds to null antagonism and 5 corresponds to the highest antagonistic effect (Fig. 1a, b). The resulting bacterial interactions (3721 numerical data) were analyzed using a statistical interactions matrix (Fig. 1c). From the 3721 interactions, 68% of the strains exhibited an antagonism degree (1–5) against one or more strains, and the remaining group null antagonism with highly susceptible phenotype (32%).

With the objective of evaluating whether the antagonistic capability is associated to antimicrobial compound production, the bacterial isolates were grown in MM9 medium, and a liquid:liquid solvent extraction of organic compounds was carried out. Different amounts of the extracts obtained from the cultures were added to filter paper disks to carry out dose–response tests using the susceptible strain of *B. coahuilensis* as bacterial lawn. Numerical data of the growth-inhibition halo around the disk showed that some extracts were capable of inhibiting the growth of the susceptible strain in a dose-dependent manner (Fig. 2a). Interestingly, almost all the extracts (~ 70%) from the isolates showed antibacterial capacity against the *B. coahuilensis* strain. Some of the extracts with major inhibition capacity were from the isolates 1B6 (*Rheinheimera* sp.), 3D8 (*Pseudomonas* sp.), and ANP5 (*Aeromonas veronii*) (Fig. 2a), that come from a mesocosm where N:P in a proportion 16:1 was added. Growth inhibition in a collection of bacteria and some of the isolates from CCB was tested using the extract of the 3D8 isolate (Fig. 2b, c). It was observed that some strains show high susceptibility to bacterial extract (*B. coahuilensis*, *B. subtilis*, *S. aureus*, CP14, CP27, CP28, CP37, CP50), while others showed resistance (such as *P. aeruginosa*, *E. coli*, AP19, ID4, 3E3, 2A4, 2A8, 4D1, CP49).

Further correlation analysis between the antagonistic effect of the bacterial isolates and the growth-inhibition activity of their extracts over the susceptible *B. coahuilensis* strain was conducted utilizing PCA analysis, observing that the bacterial population was clustered also in three correlation groups (Fig. 3). A group of isolates that showed a high antagonist activity and high growth-inhibition by their extracts (43%); the second group showed low/null antagonism and low/null growth inhibition by their extracts (32%), and the third group of isolates showed an intermediate behavior, moderate/low antagonism and moderate/low growth inhibition by their extracts (25%).

Analysis of compounds in the extracts of the bacterial isolates from the Lagunita pond of the Churince aquatic system

The inhibition of bacterial growth by the solvent-obtained extracts from cultures of the isolates from the Lagunita pond of CCB suggests that could be associated with the production of compounds with antibacterial activity; therefore, we analyzed the extracts by HPLC and GC–MS. Figure 4 shows representative HPLC chromatograms for extracts from some isolates that showed strong growth-inhibition activity (i.e., *P. aeruginosa* 1D4, *Pseudomonas* sp. 3D8, *Rheinheimera* sp. 2A9). The compounds' profiles, determined at UV 260 nm (detection of compounds with aromatic groups), showed peaks with retention times similar to synthetic CDPs cyclo(Pro-Val), cyclo(Pro-Tyr), and cyclo(Phe-Pro);

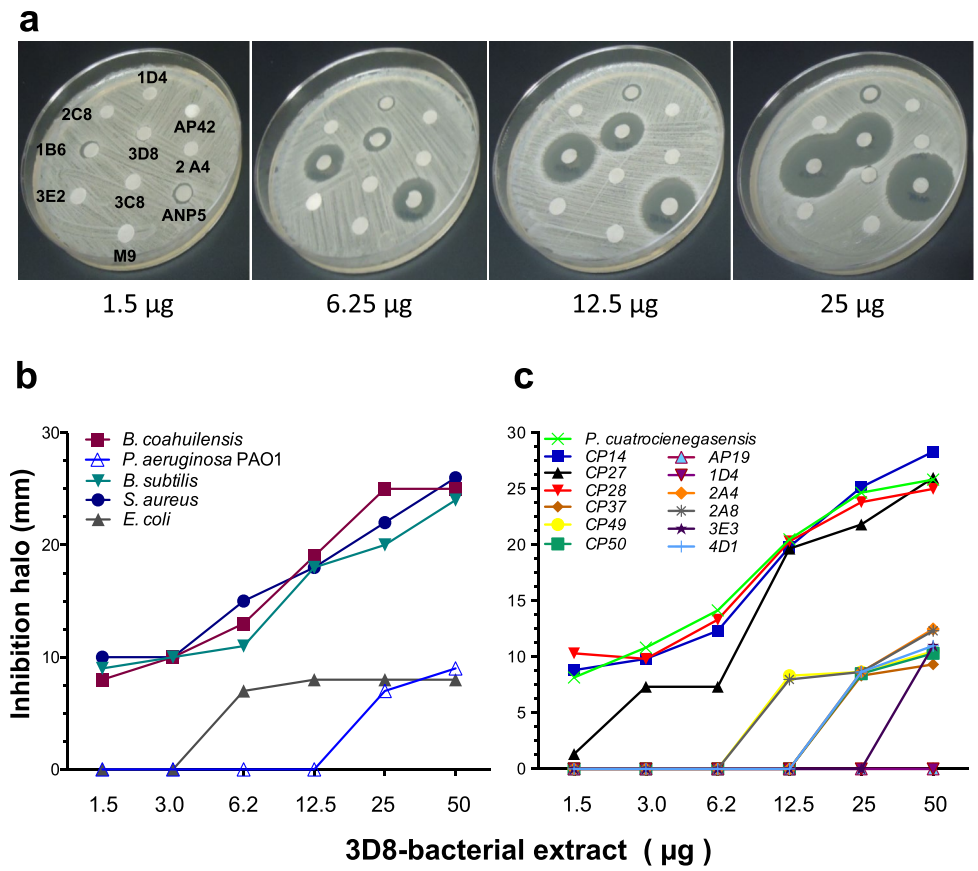


Fig. 2 Bacterial growth inhibition by extracts of the isolates from Lagunita Pond of the Cuatro Ciénegas Basin. **a** Dilutions of extracts from isolates 1D4, 2C8, AP42, 1B6, 3D8, 2A4, 3E2, 3C8, and ANP5 were dispensed on disks at indicated concentration over LB agar plates spread with susceptible strain *B. coahuilensis*. **b**, **c** After 48 h of incubation at 30 °C, growth-inhibition halos were measured. Representative data are shown for some bacterial isolates spread out (lawn) over the surface of LB plates as in **a**. Plots represent the measurements of growth-inhibition halos by the extract from the *Pseudomonas* sp. 3D8 isolate at different concentrations against the indicated strains from CCB and laboratory collection strains used as bacterial lawn; as susceptible strains (i.e., *B. coahuilensis*, *P. cuatrocienegasensis*, *B. subtilis*, *E. coli*, and *S. aureus*) and as resistant strains (i.e., *P. aeruginosa* PAO1, *P. fluorescens* AP19, *P. aeruginosa* 1D4, *Pseudomonas* sp. 3E3, *Pseudomonas* sp. 4D1, *Rheinheimera* sp. 2A4). The bacterial codes for isolates from Lagunita pond of the CCB are shown in "Materials and methods". The results are expressed as the arithmetic mean ± standard error, $n = 3$

in addition, multiple unidentified peaks were observed with strong signals that could correspond to other aromatic compounds (Fig. 4a). GC–MS analysis confirmed that the extracts derived from some of the isolates contain the CDPs cyclo(L-Pro-L-Val), cyclo(L-Pro-L-Leu), cyclo(L-Pro-L-Phe), and cyclo(L-Pro-L-Tyr); interestingly, the antibiotic 2,4-diacetylphloroglucinol (DAPG) and its intermediary phloroglucinol (PG) were also identified in some isolates such as in the 1D4 (Fig. 4b). This analysis lets us to infer that CDPs and DAPG/PG could be the main compounds with growth-inhibition effect contained in the extracts of the CCB isolates.

The presence of CDPs and DAPG/PG in extracts from isolates and the growth inhibition of their extracts were correlated by PCA analysis (Fig. 5). The PCA plot clearly shows that in the bacterial population, four bacterial clusters: (1) the CDP producers (3E3, 3E2, 2E4, 3E2, 3C7, 2A3, 2A9, 3C8, 2E2, 2A8, 2A4, 4D1, CNNP19, CNNP34, 2B7, 2C9, and 1C9) corresponding to 28% of the population; (2) the DAPG/PG producers (CNP39, DNP9, ENNP23, ENNP25, AP19, 3D8, ANP5, and 1B6) corresponding to 15% of the population; (3) the CDP/DAPG/PG producers (1A10, AP10, 1D4, BC45, 2C1, 2C2, 2C5, 2C8, 1C11, and AP42) corresponding to 15%; and (4) the null producers (neither CDP/DAPG/PG) corresponding to 43% of the bacterial population (ANNP30, 1A5, 1A8, 1A9, CP14, CNNP27, CNNP28, CP18, CP26, CP27, CP28, CP29, CP30, CP31, CP32, CP33, CP34, CP35, CP36, CP37, CP38, CP39, CP40, CP41, CP49, and CP50). PCA plot also shows that the presence of the CDPs and DAPG/PG compounds were found in 58% of the bacterial population analyzed, whose population was capable to inhibit the growth of the *B. coahuilensis* susceptible strain by their extracts. In contrast, the bacterial population that shows

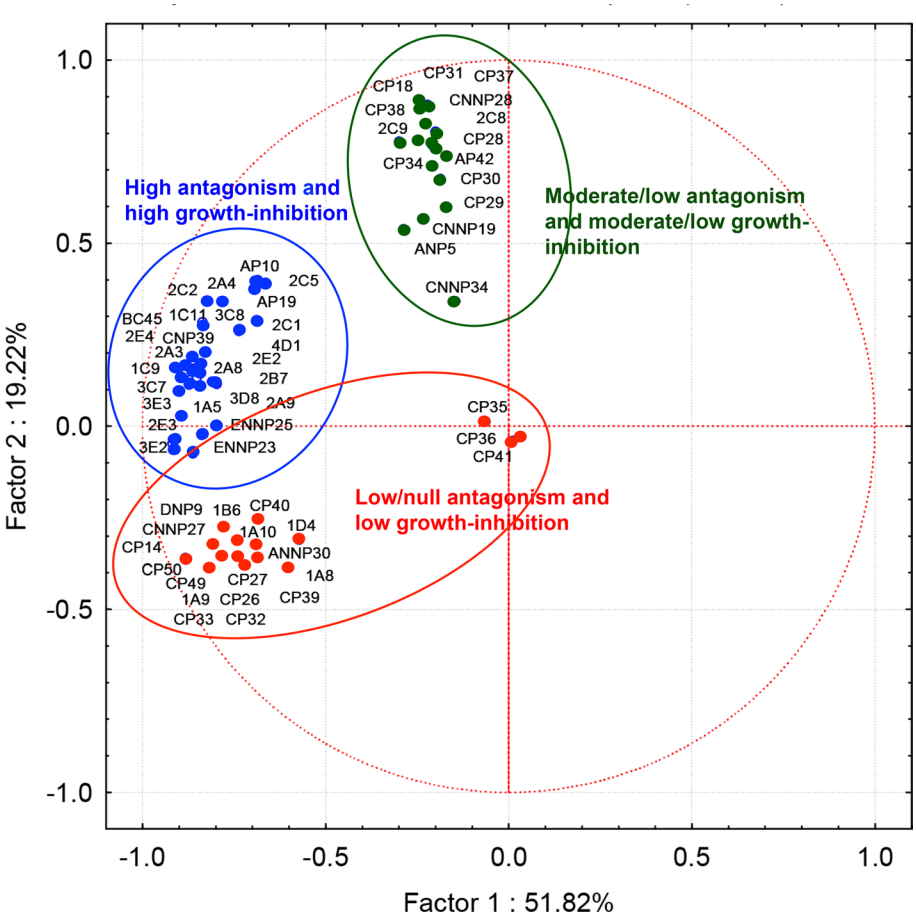


Fig. 3 PCA plot of the correlation between antagonistic effect and growth inhibition by extracts for the isolates from Lagunita Pond of the Cuatro Ciénegas Basin. The datasheet was fed with 3721 interaction data proceeding from the antagonistic assay and 61 data of the growth-inhibition assay using the bacterial extracts from all isolates of the CCB. The data were analyzed by Multivariate Exploratory Techniques, using the Principal Components and Classification Analysis (PCA) of the STATISTICA Software System 8.0. Stat Soft. Inc. Data represent the measurements done in three replicates and results are expressed as the arithmetic mean ± SD. The bacterial isolates are clustered inside circles as shown: (blue points) isolates that showed high antagonistic effect and high growth inhibition by their extracts over the susceptible *B. coahuilensis* strain; (green points) isolates that showed moderate/low antagonistic effect and moderate/low growth inhibition by their extracts; and (red points) isolates that showed low/null antagonistic effect and low growth inhibition by their extracts

lower growth inhibition by their extracts was clustered in the null producers (42%), although in this group of isolates the CDPs and DAPG/PG compounds were not identified, we do not discard them to be produced.

In other context, molecules such as AHL, involved in quorum sensing, have been implicated in bacterial population control and the dominancy in ecological niches. With the objective to explore if the antagonistic effect observed in the bacterial extracts could also be due to AHL production, we determined the induction of the violacein chromophore using the biosensor strain *C. violaceum* CV026 (McClean et al. 1997). Examples of the assays are shown in Fig. 6. Extracts from both the CDP-producer isolate 2E4 and from the DAPG-producer isolate 3D8 were unable to induce violacein production in the biosensor CV026 strain; but these isolates produced an inhibition halo over the plated biosensor strain (Fig. 6a, b). As expected, some of the isolates were capable of inducing the biosensor strain (15%), but the majority of isolates (85%) failed (Fig. 6c).

The correlation between antagonistic and extract growth-inhibition effects of the isolates from CCB is associated mainly to CDPs/DAPG/PG

The ability to antagonize the growth of the bacterial population was evaluated by PCA analysis of correlation using the response variables of the 61 bacterial isolates and as cases the data of antagonism, growth inhibition by their extracts, and the relative content of all the compounds (peaks observed in HPLC chromatograms determined at 260 nm), including the CDPs and DAPG/PG. All data were normalized using IBM SPSS statistics v22 software

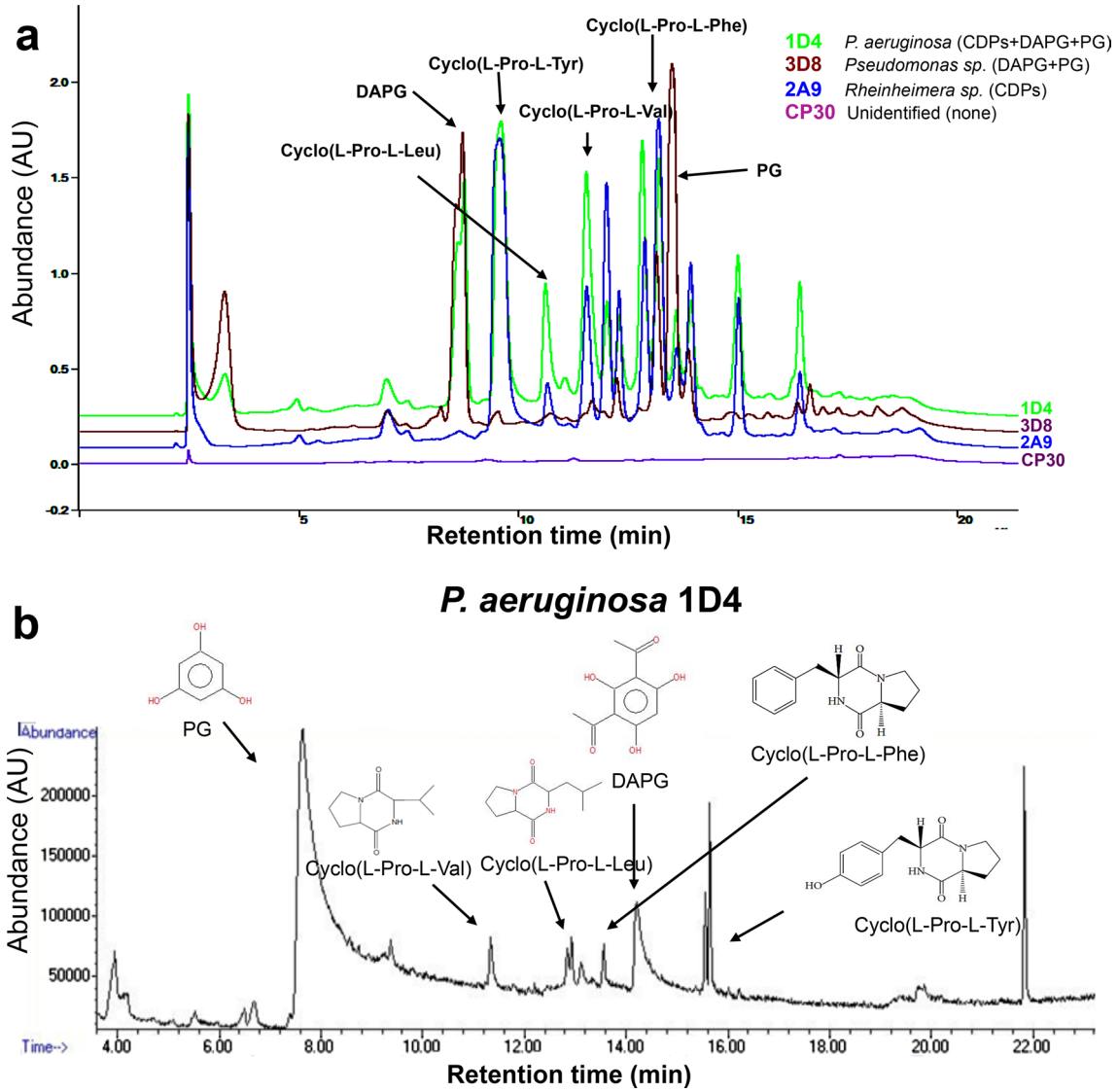


Fig. 4 Chromatographic analysis of compounds contained in the extracts of cultures of the bacterial isolates from Lagunita Pond of the Cuatro Ciénegas Basin. **a** Overlapping chromatograms corresponding to runs of four ethyl-acetate extracts of supernatants from cultures of strains isolated from Lagunita pond of CCB, analyzed by HPLC using a diode array detector at UV (260 nm). Relative abundance of the compounds is indicated by the *Y*-axis versus retention time on the *X*-axis. Arrows indicate peaks whose retention times correspond to the standard compounds: CDPs, cyclo(L-Pro-L-Val), cyclo(L-Pro-L-Leu), cyclo(L-Pro-L-Phe), and cyclo(L-Pro-L-Tyr); DAPG and PG correspond to the antibiotics 2,4-diacetylphloroglucinol and its precursor phloroglucinol, respectively. Legend shows the extracts analyzed from the 1D4, 3D8, 2A9, and CP30 bacterial isolates. **b** Representative chromatogram of the extract from cultures of the *P. aeruginosa* 1D4 isolate analyzed by GC–MS. Structure mass fragmentation patterns for the compounds identified show above 96% of probability of fragmentation ion identity with respect to the NIST-2014 library, confirming their structures with the standard compounds (Fig. S1). The relative abundance of the compounds is indicated on the *Y*-axis versus retention time on the *X*-axis. Arrows indicate peaks whose ions of mass fragmentation correspond to the compounds cyclo(L-Pro-L-Val), cyclo(L-Pro-L-Leu), cyclo(L-Pro-L-Phe), cyclo(L-Pro-L-Tyr, DAPG, and PG

as first test and later by component matrix by *k*-means using Matlab software. The PCA plot produced shows that the bacterial isolates were grouped on three statistically well-defined clusters (Fig. 7): (1) a group of isolates with high antagonistic activity, high growth-inhibition by their bacterial extracts, and that were preferably CDPs/DAPG/PG producers (55%), (2) a group with moderate/low antagonistic activity, moderate/low growth inhibition by their extracts, and that were preferably null CDPs/DAPG/PG producers (24%), and finally (3) a group of bacterial isolates with null/low antagonistic, null growth inhibition by their extracts, and that were consistently null

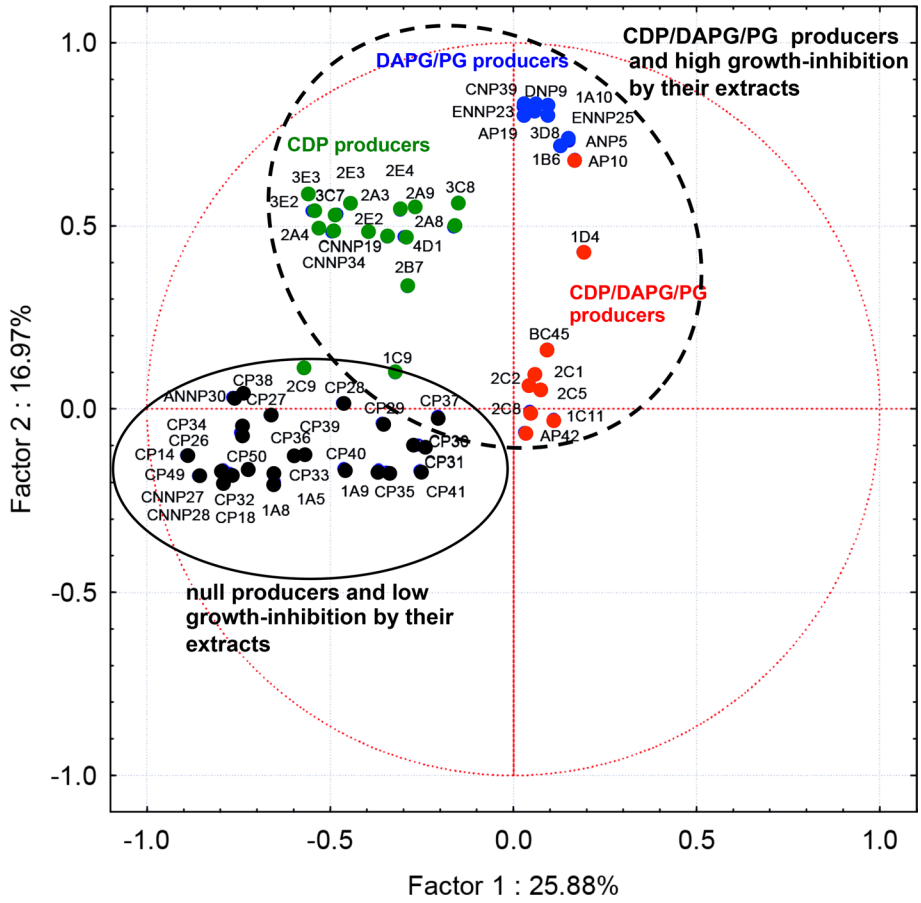


Fig. 5 PCA plot of the correlation between CDPs/DAPG/PG presence and growth inhibition by bacterial extracts of isolates from Lagunita Pond of the Cuatro Ciénegas Basin. Data of peak area of the compounds identified in bacterial extracts from the isolates including the CDPs/DAPG/PG content, determined by HPLC using a diode array detector at UV (260 nm) and the growth inhibition over the susceptible strain of *B. coahuilensis* by the bacterial extracts were analyzed using PCA with the STATISTICA Software System 8.0. Stat Soft. Inc. Data represent the measurements done in three replicates and results are expressed as the arithmetic mean ± SD. Points grouped indicate the bacterial isolates producers of: (green) CDP producers; (blue) DAPG/PG producers; (red) CDP/DAPG/PG producers and null producers (black). Solid line circle indicates bacterial population with null CDP/DAPG/PG producers and low growth-inhibition activity by their extracts. Dashed line circle clustered the CDP/DAPG/PG producers with high/moderate growth-inhibition activity by their extracts

CDPs/DAPG/PG producers (21%). From the group with the highest antagonistic activity, which also presented the highest CDP/DAPG/PG content, most of them were isolated previously to the mesocosm enrichment study; identifying 25 isolates of Gammaproteobacteria (41%) whose genus correspond to *Pseudomonas* (1C9, 2E2, 4D1, 2E3, 2E4, 3C7, AP19, ENNP23, ENNP25, 1C11, 3C8, AP42, 2C1, 2C2, 2C8, 1D4, and 2C5), *Rheinheimera* (2A3, 2A4, 2A9, and 1B6), and *Aeromonas* (3E2, 3E3, ANP5, and DNP9). The results obtained indicated that the antagonistic and growth-inhibition capabilities of the bacterial strains isolated from the Lagunita pond was associated mainly with the CDPs/DAPG/PG production; however, we do not discard that additional compounds with antibiotic properties could be involved.

Discussion

In our study, an antagonistic capacity was observed in 68% of the isolates, most of them from the sample before the mesocosm experiment started. This suggests that the "seed bank" of less competitive or cooperative strains replaced the dominant taxa (Aguirre-von-Wobeser et al. 2014, 2015; Lee et al. 2017; Ponce-Soto et al. 2015).

When we analyzed the results of the antagonistic interactions and growth inhibition to compounds contained into the culture medium extracts of each strain by PCA analysis (Fig. 3), we were able to classify the microbial community isolated from the Lagunita pond of Churince ecosystem into three groups of antagonistic capabilities: (1) high antagonistic of the bacterial population (43%), (2) low or null antagonistic (32%), and (3) an bacterial population with

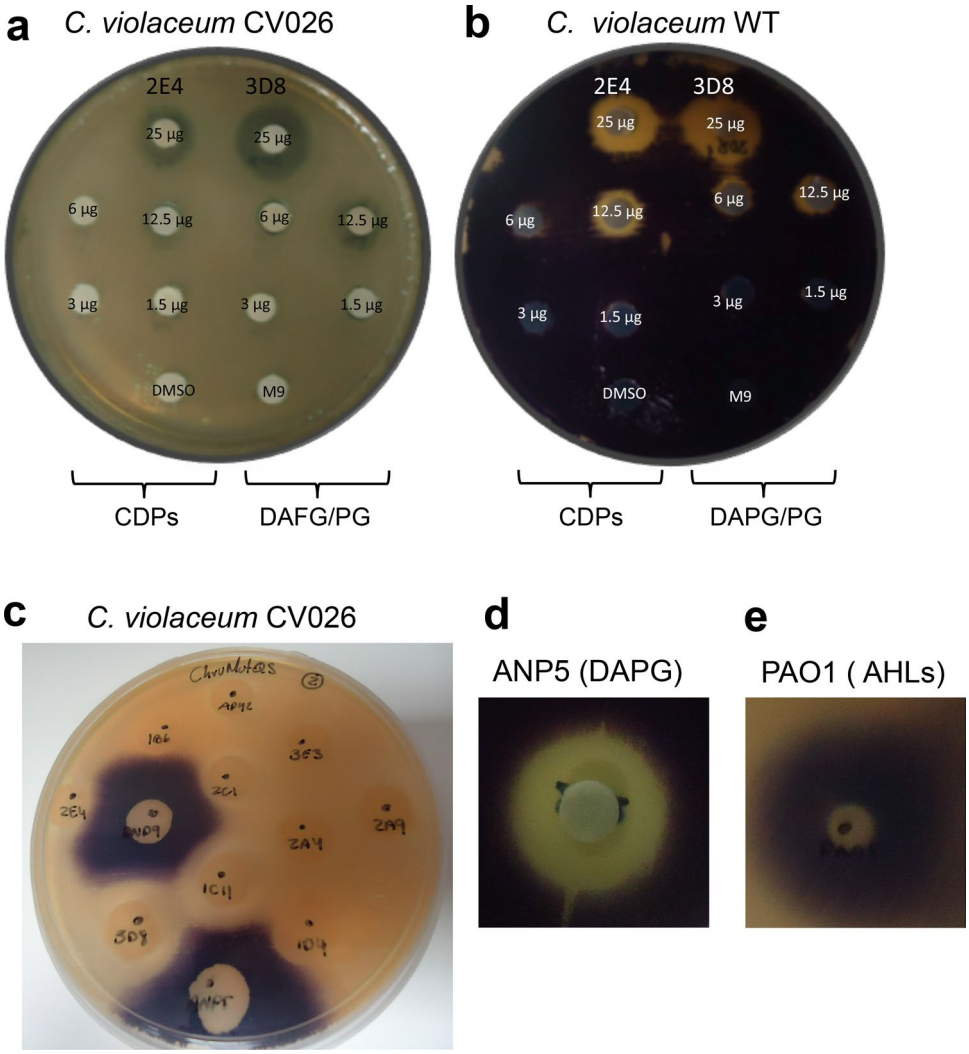


Fig. 6 Induction of violacein in the AHL-biosensor strain *Chromobacterium violaceum* CV026 by the extracts of the isolates from Lagunita Pond of the Cuatro Ciénegas Basin showing antagonistic effect. Violacein (purple pigment) induction was determined in cultures of *C. violaceum* strains plated over LB agar plates, filter paper disks containing extracts from the bacterial cultures at indicated concentrations were placed on the plate surface. The plates were incubated for 24 h at 30 °C. Afterwards, the response was determined by detection of the violacein halo produced by the biosensor strain. **a** Plate grown with the biosensor strain of *C. violaceum* CV026 (mutant unable to produce AHL) treated with extracts from the isolates *Pseudomonas* sp. 2E4 (CDPs producer) and *Pseudomonas* sp. 3D8 (DAPG producer). **b** plate grown with the *C. violaceum* wild-type (WT) strain (AHL producer) treated with same extracts in **a**. **c** Violacein induction in plates inoculated with the *C. violaceum* CV026 strain treated with extracts from antagonistic isolates; **d** control strains of DAPG producers (*Aeromonas veronii* ANP5) and **e** induction of violacein in the biosensor CV026 by *P. aeruginosa* PAO1 strain. The assays were carried out in triplicate at least three times

intermediate behavior, moderate/low antagonism (25%). The behavior shown in the first and third groups suggest that these bacterial isolates are highly competitive for resources; even if the second group with low antagonism is less competitive, it could be displaced from the community, following a kind of "prey–predator" dynamics.

Based on the results of interaction assays, we observed that bacterial extracts from the antagonist strains are capable of inhibiting growth in the *B. coahuilensis* susceptible strains in a dose-dependent manner (Fig. 2). Thus, the analyses suggest that the antagonistic effect presented by the bacterial isolates (68%) could be associated with antimicrobial compound production. Analyzing the compounds that could be involved, we found in extracts the presence of antimicrobial compounds such as CDPs and DAPG/PG. DAPG and PG, described as a class of natural products which possess broad-spectrum antibiotic properties (Yang and Cao 2012).

The CDPs/DAPG/PG presence and proportion in the extracts were correlated by PCA analysis, and four bacterial

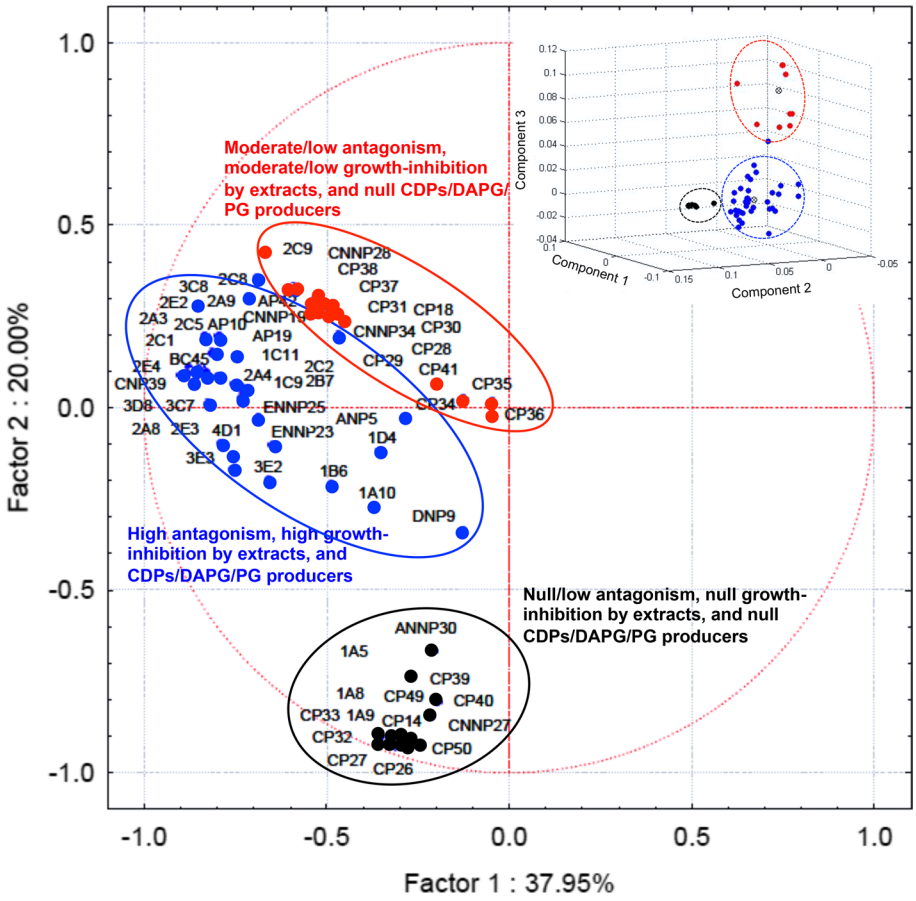


Fig. 7 PCA plot of the correlation between antagonistic effect, extracts growth-inhibition, and CDPs/DAPG/PG content in extracts of the bacterial isolates from Lagunita Pond of the Cuatro Ciénegas Basin. Data of antagonistic assay, growth inhibition over the susceptible strain of *B. coahuilensis* by the bacterial extracts, and peak area of the compound identified in bacterial extracts including the CDPs/DAPG/PG content determined by HPLC, were analyzed using PCA and *k*-means clustering. All data values were normalized in a scale of 0–1.0 and analyzed by PCA using the IBM SPSS statistics v22 as first test and after components matrix by *k*-means using the Matlab software, rendering the 3-D PCA showed as inset plot. After, data were analyzed by 2-D PCA using the STATISTICA Software System 8.0. Stat Soft. Inc. Data represent the measurements done in three replicates and results are expressed as the arithmetic mean ± SD. Points grouped within circles indicate the bacterial isolates with the following assigned characteristics: (blue) high antagonism, high growth inhibition by their extracts, and CDPs/DAPG/PG producers; (red) moderate/low antagonism, moderate/low growth inhibition by their extracts, null CDPs/DAPG/PG producers; and (black) null/low antagonism, null growth inhibition by their extracts, and null CDPs/DAPG/PG producers

clusters were clearly observed: CDP producers, DAPG/PG producers, isolates that produce both compounds, and isolates that showed null CDP/DAPG/PG production (Fig. 5). Importantly, the CDP producers were isolated mostly from mesocosm before starting the enrichment experiment (such as 1C9, 2E2, 4D1, 2E4, 2E3, 3C7, 3C8, 3E2, 3E3, 2A8, 2A3, 2A4, 2A9, 2C9, and 2B7). The DAPG/PG producers included the nutrient enrichment N:P 16:1 (CNP39, DNP9, and ANP5); N:P 75:1 (ENNP23 and ENNP25), the isolates capable of producing both types of compounds were from both before initiated and after the P enrichment (AP10, 1A10, BC45, 1C11, AP42, 2C1, 2C2, 2C5, 2C8, and 1D4); finally, the null producers, with 24 bacterial isolates (40%), belong mostly to the P-enriched treatment (Table S1).

The PCA and *k*-means clustering analysis showed a correlation between antagonism, growth inhibition of extracts, and presence of the antibacterial CDP/DAPG/PG compounds in the isolates that conformed the major proportion 55% of population (Fig. 7). Thus, the antimicrobial activity of the CDPs/DAPG/PG producers play a key role in the bacterial population control in the Lagunita pond of the Churince aquatic system.

In other context, certain CDPs can act as quorum-sensing inhibitors by blocking the signaling of regulatory LuxR-type effectors, probably by structural analogies with quorum-sensing ligands (Campbell et al. 2009; Galloway et al. 2011). That might explain the suggested agonistic/antagonistic quorum-sensing activities of CDPs in a microbial market

scenario. Thus, we used the biosensor strain *C. violaceum* CV026, a mutant strain unable to produce AHL and unable to synthetize violacein in AHL deficiency (McClean et al. 1997) to test the possible QS interference by the CDPs. Findings showed that both the extracts from the CDP-producer isolates (2E4) and DAPG-producer isolates (3D8) were unable to induce violacein production in the biosensor strain (Fig. 6). Antimicrobial activity by CDPs-producers reinforce the propose that these molecules could have a double purpose, to identify agonist microorganisms from foes and to destroy these using antagonistic interactions.

The results obtained suggest that CDPs/DAPG/PG are acting as selective or recognizing molecules from closely related strains. Even though it could be supposed that the bacterial isolates that were producers of CDPs/DAPG/PG would show the major antagonistic effect, the results indicate that bacterial antagonism in unperturbed conditions before the enrichment experiment was associated mainly with CDP production (43%), but had a minor significance to the antibiotics DAPG/PG presence (15%).

The findings suggest that the antagonistic activity as competition mechanisms of bacterial communities observed in the Lagunita pond in the Churince aquatic system of CCB is provided by the content of CDPs primarily and DAPG/PG to a lesser degree, and that the antagonistic relations among them guide the dominancy of the Gammaproteobacteria community.

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Compliance with ethical standards

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