Received: 26 February 2018 | Revised: 11 May 2018 | Accepted: 2 June 2018
DOI: 10.1002/jobm.201800087

RESEARCH PAPER

Journal of Basic Microbiology

Antimicrobial-producing *Pseudoalteromonas* from the marine environment of Panama shows a high phylogenetic diversity and clonal structure

Librada A. Atencio[1,2] | **Francesco Dal Grande**[3] | **Giannina Ow Young**[1] | **Ronnie Gavilán**[1,4,5] | **Héctor M. Guzmán**[6] | **Imke Schmitt**[3,7] | **Luis C. Mejía**[1,6] | **Marcelino Gutiérrez**[1]

[1] Centro de Biodiversidad y Descubrimiento de Drogas, Instituto de Investigaciones Científicas y Servicios de Alta Tecnología (INDICASAT-AIP), City of Knowledge, Panama, Republic of Panama

[2] Department of Biotechnology, Acharya Nagarjuna University, Guntur, India

[3] Senckenberg Biodiversity and Climate Research Centre (BiK-F), Frankfurt am Main, Germany

[4] National Center for Public Health, Instituto Nacional de Salud, Lima, Peru

[5] Environmental Management Department, Universidad San Ignacio de Loyola, Lima, Peru

[6] Smithsonian Tropical Research Institute, Ancon, Panama, Republic of Panama

[7] Department of Biological Sciences, Institute of Ecology, Evolution and Diversity, Goethe Universität Frankfurt, Frankfurt, Germany

Correspondence
Luis C. Mejía, Marcelino Gutiérrez, Centro de Biodiversidad y Descubrimiento de Drogas, INDICASAT-AIP, City of Knowledge, PO 0843–01103, Panama, Republic of Panama.
Email: lmejia@indicasat.org.pa (L.C.M); mgutierrez@indicasat.org.pa (M.G)

Funding information
National Secretariat for Science and Technology of Panama, Grant numbers: COL08-061, COL09-047; Fogarty International Center's International Cooperative Biodiversity Groups program, Grant number: TW006634; Global Environmental Fund, Grant number: GEF ID 4780–UNDP 81860; Smithsonian Tropical Research Institute; Research Funding Program Landes-Offensive zur Entwicklung Wissenschaftlich-Ökonomischer Exzellenz (LOEWE) of Hessen State Ministry for Higher Education, Research, and the Arts

Pseudoalteromonas is a genus of marine bacteria often found in association with other organisms. Although several studies have examined *Pseudoalteromonas* diversity and their antimicrobial activity, its diversity in tropical environments is largely unexplored. We investigated the diversity of *Pseudoalteromonas* in marine environments of Panama using a multilocus phylogenetic approach. Furthermore we tested their antimicrobial capacity and evaluated the effect of recombination and mutation in shaping their phylogenetic relationships. The reconstruction of clonal relationships among 78 strains including 15 reference *Pseudoalteromonas* species revealed 43 clonal lineages, divided in pigmented and non-pigmented strains. In total, 39 strains displayed moderate to high activity against Gram-positive and Gram-negative bacteria and fungi. Linkage disequilibrium analyses showed that the *Pseudoalteromonas* strains of Panama have a highly clonal structure and that, although present, recombination is not frequent enough to break the association among alleles. This clonal structure is in contrast to the high rates of recombination generally reported for aquatic and marine bacteria. We propose that this structure is likely due to the symbiotic association with marine invertebrates of most strains analyzed. Our results also show that there are several putative new species of *Pseudoalteromonas* in Panama to be described.

KEYWORDS
antimicrobial activity, linkage disequilibrium, mutation, *Pseudoalteromonas*, recombination

Abbreviations: CL, Clonal Frame; LD, Linkage disequilibrium; ST, Sequence Type.

1 | INTRODUCTION

Oceans cover about 70% of the world's surface and harbor much of the planet's biodiversity. To date many studies aiming to discover new drugs have been carried out on marine organisms [1]. Marine invertebrates including sponges, corals, mollusks, tunicates, and algae represent target organisms in such studies as they are considered a prolific source of unique bioactive molecules. There are eight drugs in the market approved by the United States Food and Drug Administration (FDA) and at least 11 natural products or their derivatives in different phases of clinical trials that are obtained from these kind of organisms [2]. However, many of these "marine invertebrate-derived" natural products, or compounds with very similar chemical scaffolds, are being re-isolated from microbial sources associated with marine invertebrates, suggesting a microbial biosynthetic origin for such compounds [3].

Microorganisms associated with marine invertebrates include fungi and a high diversity of bacteria. Recently, the chemistry of such associations has become an important research topic because of the vast array of resulting bioactive compounds. In particular, studies on the microbial communities of marine invertebrates have revealed that several groups of marine bacteria have a particularly high potential to produce bioactive compounds [4]. The most important known groups are represented by Actinomycetes [5], *Bacillus*, *Flavobacterium* [6], and *Pseudoalteromonas* [7].

The genus *Pseudoalteromonas* has caught the attention of scientists for two main reasons: first, they are widely distributed in the marine environment and they are associated with a variety of marine organisms such as corals, sponges, mollusks, fishes, tunicates as well as with seawater, sea ice, and sediments [7,8]; second, they have been shown to be capable of producing bioactive compounds with antibacterial, antifungal, algicidal and antifouling, as well as a broad profile of enzymatic activity. This ability to synthesize molecules with several bioactivities may assist *Pseudoalteromonas* species in their competition for nutrients and space and also in their symbiotic associations by providing their hosts with protection against pathogens and predators [9]. The family *Pseudoalteromonadaceae* (Gammaproteobacteria, Alteromonadales) was proposed by Ivanova et al. [8], and it is composed of three genera: *Pseudoalteromonas* [10], *Algicola* [8], and *Psychrosfaera* [11]. Phylogenetic analysis of the *Pseudoalteromonadaceae* showed that these taxa have 16S rRNA gene sequence similarity ranging from 90 to 99% [8]. *Pseudoalteromonas* is composed of two main groups forming well-supported clades in the 16S rRNA gene phylogeny [10], that is, (i) a large group of non-pigmented species, that includes the type species of the family, namely *P. haloplanktis*, and (ii) a clade of pigmented species such as the highly bioactive *P. tunicata* [11].

The production of bioactive compounds by several species of *Pseudoalteromonas* has been associated with strain pigmentation, nonetheless there are few non-pigmented strains with reported bioactivity. Pigmented species such as *P. luteaoviolacea, P. peptidolytica, P. phenolica*, and *P. piscicida* are known to have antimicrobial activity [7]. For instance, the pigmented strain *Pseudoalteromonas maricarolis* KMM 636 produces the antibacterial compounds bromo-alterochromides A and B, while *P. issachenkonii* KMM 3549, a non-pigmented strain, is known as the producer of the antifungal compound isatin (indole-2,3-dione) [11].

Pseudoalteromonas species have a distribution range occurring in both temperate and cold climate zones [12]. As biodiversity tends to increase in tropical compared to temperate areas [13], it is expected that there will be a concomitant increase in the microbial diversity associated with the tropical marine environment [14]. However, studies on the diversity of *Pseudoalteromonas* in tropical environments are largely lacking.

In this study, we explored the phylogenetic diversity of *Pseudoalteromonas* in the Panamanian marine environment taking into account the effects of recombination and mutation in its diversity and its antimicrobial capacity. Recombination and mutation interact to determine the clonality of a population. Both, recombination and mutation are key parameters in bacterial genetics. Recombination rates depend both on the ability of the DNA to enter and be incorporated into the cell, and also the ability of that genetic information to be retained by a balance of genetic drift and natural selection [15]. Panama has a biologically diverse marine ecosystem that is one of the world's marine biodiversity hotspots, including the West Caribbean and Eastern Tropical Pacific Marine Corridor. Endemic species of marine invertebrates under low to intermediate threat levels are known to occur in this region [13]. These unexplored marine environments are key targets for drug discovery research. To date, few studies have investigated microbial diversity in the marine environment of Panama and its potential as source of marine natural products.

2 | MATERIALS AND METHODS

2.1 | Strains and culture conditions

We selected 134 marine bacterial strains according to their Gram staining properties, from a previously isolated marine bacteria collection stored in the Center for Biodiversity and Drug Discovery, INDICASAT AIP, in Panama. All strains were isolated from samples collected at six sites, three located in the Caribbean Sea (Bocas del Toro, Punta Galeta, Isla

Grande), and three in the Pacific Ocean (Isla Otoque, Coiba National Park, Hannibal Bank) in Panama, between February 2009 and April 2012 (Table 1, Supporting Information Figure S1, Supporting Materials and Methods). Pure colonies were stored at −80 °C in a cryoprotectant solution of M1 broth [16] supplemented with 15% glycerol from their isolation until the present study. The strains used in this study were grown on the M1 agar medium and Marine Agar/Broth using routine procedures (Difco 2216).

2.2 | DNA extraction

Genomic DNA was extracted according to the method in Blanco-Abad et al. [17]. Briefly, one milliliter of each pure culture was grown overnight in Luria Bertani broth (Difco, USA), supplemented with seawater, at room temperature (25 °C), and subsequently centrifuged at 10,000 rpm for 2 min. The supernatant was discarded and the pellet re-suspended in 500 µl of 5% Chelex-100. These suspensions were vortexed for a few seconds and incubated at 56 °C for 20 min, boiled at 100 °C for 10 min, and then placed on ice for 2 min and centrifuged at 13,000 rpm for 5 min. Supernatants containing the DNA was transferred to a new tube and stored at −20 °C.

2.3 | Detection of *Pseudoalteromonas* strains via genus-specific PCR

A *Pseudoalteromonas*-specific PCR protocol was used to confirm the presence of *Pseudoalteromonas* within the group of selected strains. The PCR was based on the primers Eub341F and Psalt815R [9] (Supporting Information Table S1). The reaction was performed in a 50 µl reaction mixture containing 5 µl (10–30 ng μl^{-1}) of the DNA as template, each primer at a concentration of 0.5 µM, dNTPs at a concentration of 0.6 mM, 1 U of FastStart Taq DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany) and 1x buffer (Roche Diagnostics GmbH, Mannheim, Germany). See PCR conditions in Supporting Materials and Methods. PCR products were confirmed by gel electrophoresis and visualized under UV light after staining the gel with ethidium bromide (0.84 µl L^{-1}).

2.4 | PCR amplification and sequencing

We amplified and sequenced the DNA of the *Pseudoalteromonas* strains at four loci: 16S rRNA gene, and the housekeeping genes *recA*, *rpoB* and *ftsZ*.

The bacterial 16S rRNA gene was amplified from the genomic DNA using the universal eubacterial 16S rRNA primers 27F and 1492R [18] (Supporting Information Table S1). The 50 µl PCR reaction mixture contained 5 µl (~10 ng) of DNA as the template; each primer at a concentration of 0.5 µM, dNTPs at a concentration of 0.6 mM, 1 U of FastStart Taq DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany), and 1x buffer (Roche Diagnostics GmbH, Mannheim, Germany). Details about PCR conditions are presented in Supporting Information.

For the housekeeping genes the following primers were used: recA_F, recA_R, rpoB_F, rpoB_R, ftsZ_F, and ftsZ_R (Supporting Information Table S1). They were designed using the corresponding sequences derived from whole genome sequences from different strains of genus *Pseudoalteromonas* using the web server Primer4clades [19]. The 50 µl PCR reaction mixture contained 5 µl (~10 ng) DNA, each primer at the concentration of 0.5 µM, dNTPs at 0.6 µM, 1.2 U of Expand High Fidelity PCR Enzyme Mix (Roche Diagnostics GmbH, Mannheim, Germany), and 1X buffer (Roche Diagnostics GmbH, Mannheim, Germany). The PCR conditions are presented in Supporting Information. The PCR products were confirmed by gel electrophoresis and visualized under UV light after staining the gel with ethidium bromide (0.84 µl L^{-1}).

PCR amplicons were sent to Macrogen Inc. (Seoul, South Korea) for custom purification and sequencing. The primers 518F and 800R [20] for the 16S rRNA gene and recA_F, recA_R, rpoB_F, rpoB_R, ftsZ_F, ftsZ_R for protein-coding genes were used for sequencing. Raw sequences were assembled and edited using the software package Geneious R8.1 (Biomatters Ltd, Auckland, New Zealand).

2.5 | Sequence data analyses

To confirm that all the strains studied belonged to the genus *Pseudoalteromonas* we used the 16S Biodiversity tool in Geneious R8.1 (Biomatters Ltd, Auckland, New Zealand), which compares 16S rRNA consensus sequences against RDP (Ribosomal Database Project) [21]. In addition, sequences for each gene (16S rRNA, *recA*, *rpoB*, and *ftsZ*) were also compared to the non-redundant database of sequences deposited in GenBank using the *blastn* algorithm and keeping a maximum of 100 hits per query sequence. All the sequences were submitted to GenBank database, the accession numbers for each sequence are shown in Table 1.

2.6 | Phylogenetic analyses

Multiple alignments of sequences of the *Pseudoalteromonas* strains were performed with MAFFT v7.017 [22].

A dataset of 16S rRNA genes was created including, *Pseudoalteromonas* strains sequences from Panama (78 sequences) and *Pseudoalteromonas* types and reference strains (49 sequences, Supporting Information Table S10) to reconstruct a 16S rRNA gene maximum likelihood phylogeny using RAxML [23] with the GTR-GAMMA

TABLE 1 Isolation source and collection sites of Pseudoalteromonas strains of Panama and type and reference strains

CL	Strain	Isolation source	Host Species	Year	Collection site	GenBank accession numbers			
						16s rRNA	recA	rpoB	ftsZ
	Non-pigmented								
1	CO6016X	Octocoral	*Millepora imbricata*	2010	Veraguas: Coiba National Park: Canal del Sur	KU213110	KU213265	KU213343	KU213187
	CO1916	Sponge	Porifera	2010	Veraguas: Coiba National Park: Canal del Sur	KU213090	KU213245	KU213323	KU213167
	CO2020	Oyster	*Pinctada mazatlantica*	2010	Veraguas: Coiba National Park: Frjolito	KU213092	KU213247	KU213325	KU213169
	CO1816	Stony coral	*Tubastrea* sp.	2010	Veraguas: Coiba National Park: Bajo 20	KU213100	KU213255	KU213333	KU213177
	CO1320	Seawater	N/A	2010	Veraguas: Coiba National Park: Twist Peak	KU213097	KU213252	KU213330	KU213174
	CO3417	Hydrozoan	Hydrozoa	2010	Veraguas: Coiba National Park: Frjolito	KU213095	KU213250	KU213328	KU213172
	CO6016Y	Octocoral	*Millepora imbricata*	2010	Veraguas: Coiba National Park: Canal del Sur	KU213111	KU213266	KU213344	KU213188
2	BO63	Octocoral	*Pseudopterogorgia acerosa*	2009	Bocas del Toro: Isla Colón	KU213089	KU213244	KU213322	KU213166

(Continues)

TABLE 1 (Continued)

CL	Strain	Isolation source	Host Species	Year	Collection site	GenBank accession numbers			
						16s rRNA	recA	rpoB	ftsZ
3	IG343	Octocoral	*Pterogorgia anceps*	2011	Colón: Isla Grande	KU213098	KU213253	KU213331	KU213175
4	IG13X	Octocoral	*Muriceopsis* sp.	2011	Colón: Isla Grande	KU213113	KU213268	KU213346	KU213190
	IG263	Octocoral	*Pterogorgia anceps*	2011	Colón: Isla Grande	KU213091	KU213246	KU213324	KU213168
5	CO2118Y	Sponge	Porifera	2010	Veraguas: Coiba National Park: Canal del Sur	KU213136	KU213291	KU213369	KU213213
	CO2118X	Sponge	Porifera	2010	Veraguas: Coiba National Park: Canal del Sur	KU213093	KU213248	KU213326	KU213170
6	CO6416X	Stony Coral	Scleractinia	2010	Veraguas: Coiba National Park: Bajo 20	KU213099	KU213254	KU213332	KU213176
	IG153	Octocoral	*Pterogorgia anceps*	2011	Colón: Isla Grande	KU213096	KU213251	KU213329	KU213173
	CO327W	Octocoral	*Pacifigorgia bayeri*	2009	Veraguas: Coiba National Park: Roca Hacha	KU213101	KU213256	KU213334	KU213178
7	CO18B1	Octocoral	*Leptogorgia* sp.	2012	Veraguas: Coiba National Park: Banco Hannibal	KU213094	KU213249	KU213327	KU213171
	IG13Y	Octocoral	*Muriceopsis* sp.	2011	Colón: Isla Grande	KU213125	KU213280	KU213358	KU213202

(Continues)

TABLE 1 (Continued)

CL	Strain	Isolation source	Host Species	Year	Collection site	GenBank accession numbers			
						16s rRNA	recA	rpoB	ftsZ
8	IG23	Octocoral	*Muriceopsis* sp.	2011	Colón: Isla Grande	KU213138	KU213293	KU213371	KU213215
9	IG633	Octocoral	*Eunicea* sp.	2011	Colón: Isla Grande	KU213138	KU213294	KU213372	KU213216
	IG163	Octocoral	*Pterogorgia anceps*	2011	Colón: Isla Grande	KU213139	KU213295	KU213373	KU213217
10	CO69X	Octocoral	*Pacifigorgia firma*	2009	Veraguas: Coiba National Park: Barco Quebrado	KU213114	KU213269	KU213347	KU213191
11	CO109Y	Octocoral	*Eugorgia daniana*	2009	Veraguas: Coiba National Park: Roca Hacha	KU213115	KU213270	KU213348	KU213192
	CO109X	Octocoral	*Eugorgia daniana*	2009	Veraguas: Coiba National Park: Roca Hacha	KU213126	KU213281	KU213359	KU213203
12	CO3318Y	Sponge	Porifera	2010	Veraguas: Coiba National Park: Isla Afuera Norte	KU213118	KU213273	KU213351	KU213195
13	CO5217X	Stony coral	Scleractinia	2010	Veraguas: Coiba National Park: Canal del Sur	KU213124	KU213279	KU213357	KU213201
	CO5217Y	Stony coral	Scleractinia	2010	Veraguas: Coiba National Park: Canal del Sur	KU213121	KU213276	KU213354	KU213198

(Continues)

TABLE 1 (Continued)

CL	Strain	Isolation source	Host Species	Year	Collection site	GenBank accession numbers			
						16s rRNA	recA	rpoB	ftsZ
14	CO6420X	Sea urchin	*Eucidaris* sp.	2010	Veraguas: Coiba National Park: Canal del Sur	KU213120	KU213275	KU213353	KU213197
	CO6420Y	Sea urchin	*Eucidaris* sp.	2010	Veraguas: Coiba National Park: Canal del Sur	KU213122	KU213277	KU213355	KU213199
	CO4620Y	Stony coral	*Porites panamensis*	2010	Veraguas: Coiba National Park: Roca Hacha	KU213119	KU213274	KU213352	KU213196
15	CO317X	Sponge	Porifera	2010	Veraguas: Coiba National Park: La Lavadora	KU213116	KU213271	KU213349	KU213193
	CO4620X	Stony coral	*Porites panamensis*	2010	Veraguas: Coiba National Park: Roca Hacha	KU213117	KU213272	KU213350	KU213194
	CO317Y	Sponge	Porifera	2010	Veraguas: Coiba National ParkLa Lavadora	KU213123	KU213278	KU213356	KU213200
16	IG183	Octocoral	*Pterogorgia anceps*	2011	Colón: Isla Grande	KU213104	KU213259	KU213337	KU213181
17	CO311X	Octocoral	*Muricea* sp.	2009	Veraguas: Coiba National Park: Roca Hacha	KU213108	KU213263	KU213341	KU213185

(Continues)

TABLE 1 (Continued)

CL	Strain	Isolation source	Host Species	Year	Collection site	GenBank accession numbers			
						16s rRNA	recA	rpoB	ftsZ
	CO314	Octocoral	*Pacifigorgia firma*	2009	Veraguas: Coiba National Park: Barco Quebrado	KU213107	KU213262	KU213340	KU213184
	CO311Y	Octocoral	*Muricea* sp.	2009	Veraguas: Coiba National Park: Roca Hacha	KU213102	KU213257	KU213335	KU213179
18	IG253	Octocoral	*Pterogorgia anceps*	2011	Colón: Isla Grande	KU213106	KU213261	KU213339	KU213183
	CO327Y	Octocoral	*Pacifigorgia bayeri*	2009	Veraguas: Coiba National Park: Roca Hacha	KU213105	KU213260	KU213338	KU213182
19	CO272Y	Octocoral	*Pacifigorgia catedralensis*	2009	Veraguas: Coiba National Park: Catedral	KU213137	KU213292	KU213370	KU213214
	CO272X	Octocoral	*Pacifigorgia catedralensis*	2009	Veraguas: Coiba National Park: Catedral	KU213112	KU213267	KU213345	KU213189
20	CO5520Y	Zoanthids	Zoantharia	2010	Veraguas: Coiba National Park: Catedral	KU213109	KU213264	KU213342	KU213186
	CO5520X	Zoanthids	Zoantharia	2010	Veraguas: Coiba National Park: Catedral	KU213103	KU213258	KU213336	KU213180

(Continues)

TABLE 1 (Continued)

CL	Strain	Isolation source	Host Species	Year	Collection site	GenBank accession numbers			
						16s rRNA	recA	rpoB	ftsZ
21	CO133X	Octocoral	*Pacifigorgia smitsoniana*	2009	Veraguas: Coiba National Park: Catedral	KU213127	KU213282	KU213360	KU213204
22	CO302Y	Octocoral	*Psammogorgia* sp.	2009	Veraguas: Coiba National Park: Roca Hacha	KU213129	KU213284	KU213362	KU213206
	CO253X	Octocoral	*Pacifigorgia cairnsi*	2009	Veraguas: Coiba National Park: Roca Hacha	KU213128	KU213283	KU213361	KU213205
	CO253Y	Octocoral	*Pacifigorgia cairnsi*	2009	Veraguas: Coiba National Park: Roca Hacha	KU213131	KU213286	KU213364	KU213208
	CO331Y	Octocoral	*Leptogorgia tabogilla*	2009	Veraguas: Coiba National Park: Roca Hacha	KU213130	KU213285	KU213363	KU213207
	CO331X	Octocoral	*Leptogorgia tabogilla*	2009	Veraguas: Coiba National Park: Roca Hacha	KU213133	KU213288	KU213366	KU213210
	CO302X	Octocoral	*Psammogorgia* sp.	2009	Veraguas: Coiba National Park: Roca Hacha	KU213132	KU213287	KU213365	KU213209

(Continues)

TABLE 1 (Continued)

CL	Strain	Isolation source	Host Species	Year	Collection site	GenBank accession numbers			
						16s rRNA	recA	rpoB	ftsZ
23	CO4819Y	Bryozoan	Bryozoa	2010	Veraguas: Coiba National Park: Canal del Sur	KU213134	KU213289	KU213367	KU213211
	CO4819X	Bryozoan	Bryozoa	2010	Veraguas: Coiba National Park: Canal del Sur	KU213135	KU213290	KU213368	KU213212
24	*P. lipolytica* SCSIO 04301	Marine sediment	N/A	N/D	South China Sea	KC894020	JDVB01000006.1	NZ_JDVB00000000	JDVB01000006
25	*P. atlantica* TB41	Sponge	*Anoxycalyx joubini*	N/D	Antartic: Tethys Bay	Nté	NZ_AUTH01000088	NZ_AUTH00000000	NZ_AUTH01000024
	P. undina NCIMB 2128^T	Seawater	N/A	N/D	USA: Coast of Northern Carolina	X82140	NZ_AHCF02000038.1	NZ_AHCF00000000	AHCF02000013
26	*P. agarivorans* S816 S816	Seawater	N/A	N/D	Denmark	APME01000086	NZ_APME01000005.1	NZ_APME01000034.1	NZ_APME01000078.1
	P. marina mano4^T	Flat tidal sediment	N/A	N/D	Korea: Chung-Nam: Dae-Chun	AY563031	NZ_AHCB02000001.1	NZ_AHCB00000000	NZ_AHCB02000001.1
27	*P. haloplanktis* TAC 125	Seawater	N/A	N/D	French: Antarctic station Dumont d'Urville: Terre Adélie	NR_102834	NZ_JH650748.1	NZ_JH650753.1	NZ_JH650743
28	*P. arctica* A 37-1-2^T	Seawater	N/A	N/D	Spitzbergen, Norway	DQ787199	NZ_AHBY02000046.1	NZ_AHBY02000006.1	AHBY02000119
	Pigmented								

(Continues)

TABLE 1 (Continued)

CL	Strain	Isolation source	Host Species	Year	Collection site	GenBank accession numbers			
						16s rRNA	recA	rpoB	ftsZ
30	CO325X	Octocoral	*Muricea austera*	2009	Veraguas: Coiba National Park: Roca Hacha	KU213064	KU213219	KU213297	KU213141
	GA327	Octocoral	*Muriceopsis sulphurea*	2009	Colón: Punta Galeta	KU213068	KU213223	KU213301	KU213145
31	GA189	Octocoral	*Muriceopsis sulphurea*	2009	Colón: Punta Galeta	KU213069	KU213224	KU213302	KU213146
32	GA20	Sponge	Porifera	2009	Colón: Punta Galeta	KU213065	KU213220	KU213298	KU213142
	GA204	Octocoral	*Muriceopsis sulphurea*	2009	Colón: Punta Galeta	KU213067	KU213222	KU213300	KU213144
	GA123	Sponge	Porifera	2009	Colón: Punta Galeta	KU213066	KU213221	KU213299	KU213143
33	BA24	Octocoral	*Briareum asbestimum*	2009	Bocas del Toro: Isla Colón	KU213070	KU213225	KU213303	KU213147
35	OT59	Octocoral	*Leptogorgia* alba	2009	Panama: Isla Otoque	KF880834	KU213296	KU213374	KU213218
36	CO34	Octocoral	*Psammogorgia* sp.	2009	Veraguas: Coiba National Park: Roca Hacha	KU213081	KU213236	KU213314	KU213158
37	BA54	Octocoral	*Briareum asbestimum*	2009	Bocas del Toro: Isla Colón	KU213071	KU213226	KU213304	KU213148
38	CO4016X	Octocoral	*Pacifigorgia rubicunda*	2010	Veraguas: Coiba National Park: Roca Hacha	KU213077	KU213232	KU213310	KU213154
39	BA77	Octocoral	*Briareum asbestimum*	2009	Bocas del Toro: Isla Colón	KU213072	KU213227	KU213305	KU213149

(Continues)

TABLE 1 (Continued)

CL	Strain	Isolation source	Host Species	Year	Collection site	GenBank accession numbers			
						16s rRNA	recA	rpoB	ftsZ
	CO342X	Octocoral	*Muricea* sp.	2009	Veraguas: Coiba National Park: Roca Hacha	KU213074	KU213229	KU213307	KU213151
40	BO104	Octocoral	*Plexaura* sp.	2009	Bocas del Toro: Isla Colón	KU213084	KU213239	KU213317	KU213161
	BO105	Octocoral	*Plexaura* sp.	2009	Bocas del Toro: Isla Colón	KU213079	KU213234	KU213312	KU213156
41	CO18A2	Octocoral	*Leptogorgia* sp.	2012	Veraguas: Coiba National Park: Banco Hannibal	KU213073	KU213228	KU213306	KU213150
	CO19A1	Octocoral	*Eugorgia rubens*	2012	Veraguas: Coiba National Park: Banco Hannibal	KU213082	KU213237	KU213315	KU213159
	CO19A2	Octocoral	Octocorallia	2012	Veraguas: Coiba National Park: Banco Hannibal	KU213083	KU213238	KU213316	KU213160
	CO26A	Red algae	Rhodophyta	2012	Veraguas: Coiba National Park: Banco Hannibal	KU213085	KU213240	KU213318	KU213162
	CO19B	Octocoral	Octocorallia	2012	Veraguas: Coiba National Park: Banco Hannibal	KU213088	KU213243	KU213321	KU213165

(Continues)

TABLE 1 (Continued)

CL	Strain	Isolation source	Host Species	Year	Collection site	GenBank accession numbers			
						16s rRNA	recA	rpoB	ftsZ
	CO18A3	Octocoral	*Leptogorgia* sp.	2012	Veraguas: Coiba National Park: Banco Hannibal	KU213086	KU213241	KU213319	KU213163
	CO19A3	Octocoral	Octocorallia	2012	Veraguas: Coiba National Park: Banco Hannibal	KU213087	KU213242	KU213320	KU213164
42	CO348	Octocoral	*Leptogorgia cofrini*	2009	Veraguas: Coiba National Park: Catedral	KU213080	KU213235	KU213313	KU213157
43	GA16	Sponge	*Amphimedon compressa*	2009	Colón: Punta Galeta	KU213078	KU213233	KU213311	KU213155
	GA302	Sponge	*Niphates erecta*	2009	Colón: Punta Galeta	KU213075	KU213230	KU213308	KU213152
	GA303	Sponge	*Amphimedon compressa*	2009	Colón: Punta Galeta	KU213076	KU213231	KU213309	KU213153
28	*P. fuliginea* KMM 216^T	Seawater	N/A	N/D	Mediterranean Sea near Nice	AF082563	NZ_JJNZ01000020.1	NZ_JJNZ01000024	NZ_JJNZ01000031.1
29	*P. tunicata* D2^T	Tunicate	*Ciona intestinalis*	N/D	Sweden: Gullmarsfjorden	Z25522	NZ_AAOH01000002.1	NZ_CH959304	NZ_AAOH01000003.1
30	*P. ruthenica* CP76	Salterns	N/A	N/D	Spain: Huelva: Isla Cristina	NZ_AOPM01000107	NZ_AOPM01000101.1	NZ_AOPM00000000	NZ_AOPM01000081
33	*P. spongiae* UST010723-006^T	Sponge	*Mycale adhaerens*	N/D	Hong Kong	AY769918	NZ_AHCE02000011.1	NZ_AHCE02000007.1	NZ_AHCE02000018.1
34	*P. luteoviolacea* 2ta16	Coral	*Montastrea annularis*	N/D	USA: Florida Keys	AUSV01000047	JQ280429	NZ_AUSV01000089	AUSV01000003
34	*P. rubra* ATCC 29570^T	Seawater	N/A	N/D	France: Nice	X82147	NZ_AHCD02000129.1	NZ_AHCD02000161.1	AHCD02000036

(Continues)

TABLE 1 (Continued)

CL	Strain	Isolation source	Host Species	Year	Collection site	GenBank accession numbers			
						16s rRNA	recA	rpoB	ftsZ
36	*P. flavipulchra* JG1	fish (turbot)	*Scophthalmus maximus*	N/D	China: Quingdao	GU325751	NZ_JH650748.1	NZ_JH650753.1	NZ_JH650743
37	*P. piscicida* ATCC 15057^T	Seawater (Red tide)	N/A	N/D	USA: Florida	AB090232	NZ_AHCC02000126.1	NZ_KB907389.1	NZ_KB907371

CL, Clonal Lineage; N/D, No Data; N/A, Does not apply; T, Type strain.

substitution model on CIPRES portal (https://www.phylo.org/). Representative sequences from closely related taxa (*Algicola bacteriolitica* IAM 14595^T and *Psychromonas aquimarina* ATCC BAA 1526) were used as out-groups. From here "reference strains" refers to *Pseudoalteromonas* strains that were included in the study and are not type strain of the genus or species.

Four-locus phylogeny using ClonalFrame: Four datasets of *Pseudoalteromonas* strains were used: 1) a dataset with all sequences from this study (78 sequences), 2) the corresponding subsets of non-pigmented (52 sequences), and 3) pigmented (26 sequences) strains. In order to relate strains of this study to published *Pseudoalteromonas* reference strains we additionally produced a dataset 4) including all sequences from this study and 15 type/reference strains of *Pseudoalteromonas* for which at least three of the sequenced loci were available (93 sequences).

For a reliable inference of phylogenetic relationships among bacteria strains, the role of recombination was taken into account. For this purpose, we used the software ClonalFrame v1.2 [24] (Details about this method are presented in Supporting Information). Three independent runs of ClonalFrame were performed per dataset, each consisting of 100,000 Markov Chain Monte Carlo (MCMC) iterations, with an initial burn-in of 50,000 generations, sampling at each 100 steps. To assess the relative contribution of recombination and mutation events to each dataset, we calculated *r/m* and *rho/theta* statistics [25,26]. We checked the convergence of the MCMC in different runs using the Gelman-Rubin test [27]. As convergence was verified, we built a 50% majority-rule consensus tree for dataset 1, summarizing all 3,003 trees of the posterior sample of the three runs.

To explore clonal relationships among isolates and reconstruct ancestral nodes in the phylogeny we built a DOT graph using the program Graphviz neato (https://www.graphviz.org/) on all 3003 trees of the ClonalFrame posterior sample of dataset 4. Phylogenetic and clonal network analyses were performed using the ClonalFrame package. To taxonomically annotate the reconstructed clonal lineages, 16S rRNA gene sequences of each clonal lineage of the network were aligned against the SILVA reference database using SINA with a 98% similarity threshold [28].

2.7 | Linkage disequilibrium (LD)

To gain insights into the phylogenetic relationships and origin of the *Pseudoalteromonas* isolates of Panama, we estimated linkage disequilibrium (LD). LD between loci was assessed using two indices of association, namely I_A index of association [29] and the standardized index

rbarD [30] derived with the program Multilocus v1.3b [30]. Unlike I_A, rbarD is independent of the number of loci analyzed, ranging from 0 (panmixia) to 1 (clonality) and allows comparisons among datasets. We inferred the statistical significance of these indices using 1000 randomized datasets under the null hypothesis of panmixia. We calculated indices of association for two types of datasets: i) a "population" dataset in which all samples were retained, and ii) sequence-type dataset (STs), in which identical sequences were collapsed into a single, distinct multilocus genotype (Supporting Information Tables S4–S9). *Pseudoalteromonas* structure was categorized as i) panmictic or clonal, if the value of rbarD approached always either 0 or 1, respectively, or ii) epidemic, if rbarD was significantly positive when calculated from the "population" dataset, but close to zero when calculated from STs [29–32].

2.8 | Screening for antibacterial and antifungal activity

The *Pseudoalteromonas* strains were screened for antibacterial activity following the protocol by Castillo *et al.* [33], except for the use of the target strains at determined concentrations. As target strains we used *Bacillus subtilis* subsp. *subtilis* ATCC 6051, *Bacillus pumilus* ATCC 7061, *Vibrio coralliilyticus* ATCC BAA 450, *Pseudoalteromonas haloplanktis* ATCC 14393, *Acinetobacter baumanii* ATCC 19606, *Staphylococcus aureus* ATCC 43300, *Pseudomonas aeruginosa* ATCC 10145, and *Escherichia coli* ATCC 10536. Briefly, the method consisted of spreading 0.5 McFarland (1.5×10^8 CFU ml^{-1}) suspensions of each bacterial tester on squared petri dishes with M1 agar. After 20 min, a loop-full (10 µl) of pure cultures of marine bacteria was inoculated as cumulus. Following incubation for 18–24 h at 30 °C, except for *Pseudoalteromonas haloplanktis* ATCC 14393 that was incubated at room temperature (25 °C). After this period, the plates were examined for the formation of inhibition zones around the bacterial spot of marine bacteria indicating the production of secondary metabolites with antibacterial activity. Activity was considered to occur when the diameter of the zone of inhibition was at least 2 mm greater than the diameter of the colony formed by the potential producer [34].

To test for antifungal activity, we used the strains *Candida albicans* ATCC 10231 and *Aspergillus fumigatus* ATCC 1028. *Candida albicans* was analyzed using the procedure described above for bacteria. To test for activity against *A. fumigatus* ATCC 1028, a conidial solution was prepared in tubes containing saline solution tubes (0.85% w/v NaCl) to achieve an optical density of 0.09–0.11 ($0.6–5 \times 10^6$ CFU ml^{-1}); after spreading the tester on M1 plates, marine bacteria were inoculated as a cumulus, following an incubation at 30 °C for 48 h to 72 h. Each plate was checked for the formation of inhibition zones.

3 | RESULTS

3.1 | Detection of *Pseudoalteromonas*

Using *Pseudoalteromonas*-specific primers [9], we detected 84 putative *Pseudoalteromonas* strains from a subset of 134 Gram negative bacteria available in the marine bacteria collection of INDICASAT AIP. Subsequently, by comparing DNA sequences of genes encoding for the 16S rRNA (1400–1500 bp), the DNA and recombination repair protein (*recA,* 497 bp), the beta subunit of RNA polymerase (*rpoB,* 657 bp) and the cell division gene *ftsZ* (469 bp) against the RDP and GenBank databases, we retained 78 strains that truly belonged to the genus within the range of 99–100% identity (Table 1). The remaining six strains were not included because were identified as members of others bacterial genera (*Ruegeria*, *Halomonas*, *Serinicoccus* and *Vibrio*).

3.2 | Phylogenetic analyses

3.2.1 | 16S rRNA gene phylogenetic reconstruction

Three main groups of *Pseudoalteromonas* strains were identified in the maximum likelihood (ML) phylogenetic reconstruction based on 16S rRNA gene sequences (Fig. 1). The first group (Fig. 1–I) was composed of most of non-pigmented *Pseudoalteromonas* reference strains (for details see Supporting Information Table S9), and included *P. haloplanktis* (strains ATCC 14393^T and TAC125), type species of the *Pseudoalteromonadaceae*. The second group (Fig. 1–II) included several clades composed of strains isolated from different octocoral species and closely related to *P. arabiensis* k53^T with high bootstrap support (BS 100%). In this group, a few strains from the Panamanian localities Punta Galeta and Coiba National Park resulted to be closely related to the type strain of *P. ruthenica* (KMM300^T, BS 100%). The recently described *Pseudoalteromonas shioyasakiensis* SE3^T was also found to be in the non-pigmented clade, but with a low bootstrap support. The remaining strains of *Pseudoalteromonas* from the marine environment of Panama were found to form a distinctive clade without close relationship to type strains of previously described *Pseudoalteromonas*. The third clade (Fig. 1–III) included two well-supported (BS > 80%) monophyletic groups of pigmented *Pseudoalteromonas* strains from different sites of Panama. The phylogeny shows that this group is closely related to species such as *P. flavipulchra* (NCIMB 2033$^{T)}$, *P. maricarolis* (CIP 106859^T), *P. peptidolytica* (F12-50-A1^T), *P. piscicida* (ATCC 15057^T), and *P. spongiae* (UST010723-006^T).

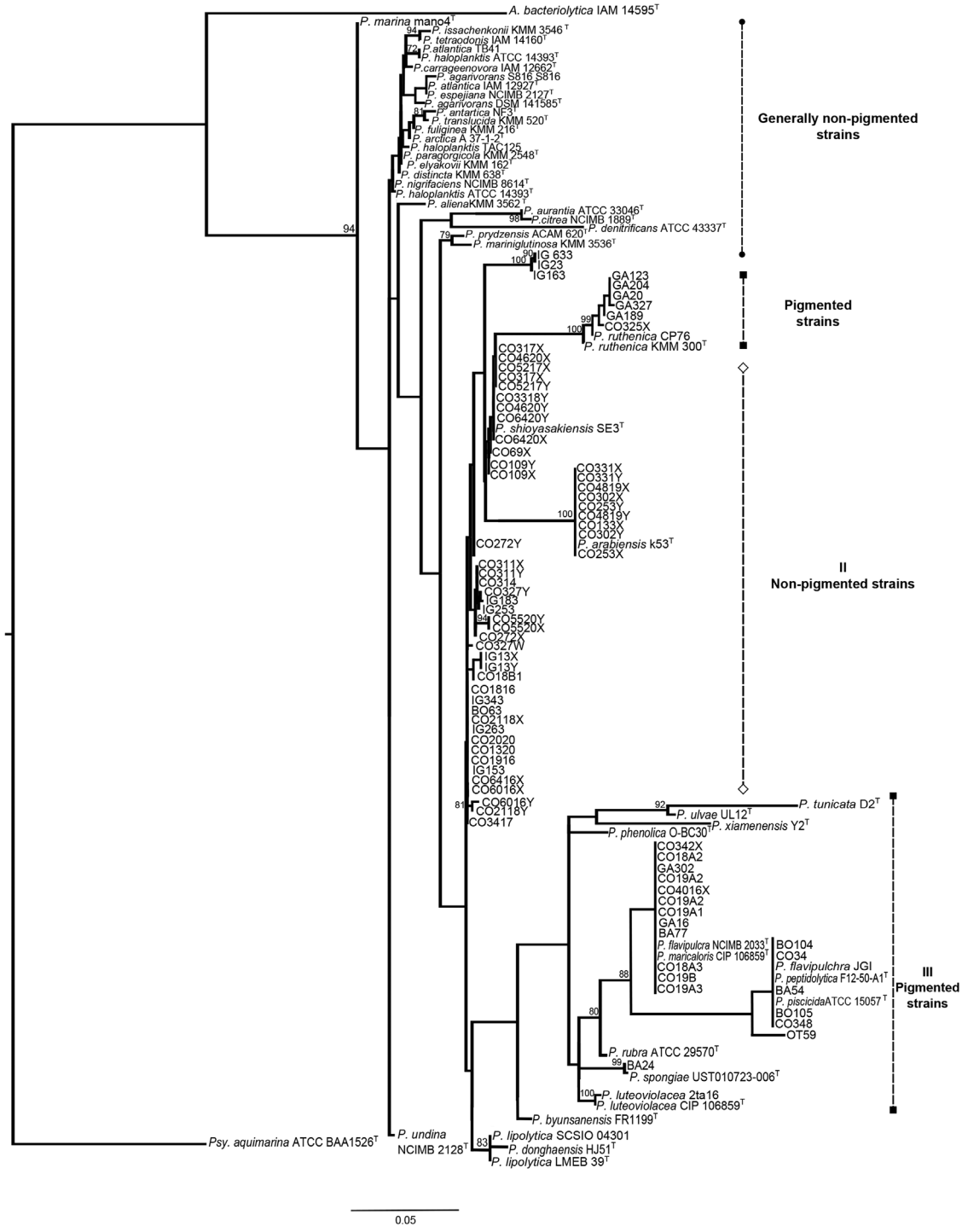

FIGURE 1 Phylogenetic reconstruction of *Pseudoalteromonas* strains from Panama based on 16S rRNA gene. The sequence dataset included *Pseudoalteromonas* strains from the marine environment of Panama and *Pseudoalteromonas* types and reference strains (Supporting Information Table S10). The tree was constructed using maximum likelihood algorithm RAxML [23]. Bootstrap values grater than 70 are shown next to its respective branch. Closely related taxa, as *Algicola bacteriolytica* and *Psychromonas aquimarina* were used as out-groups

3.2.2 | Four-locus phylogeny using ClonalFrame

We used ClonalFrame (CF) to infer the phylogenetic relationships of the isolates based on the concatenation of sequences from the genes 16S rRNA, recA, rpoB, and ftsZ. The CF phylogenetic analysis resolved two well-supported (posterior probability (PP) > 95%) monophyletic groups corresponding to non-pigmented and pigmented *Pseudoalteromonas* lineages, respectively (Fig. 2).

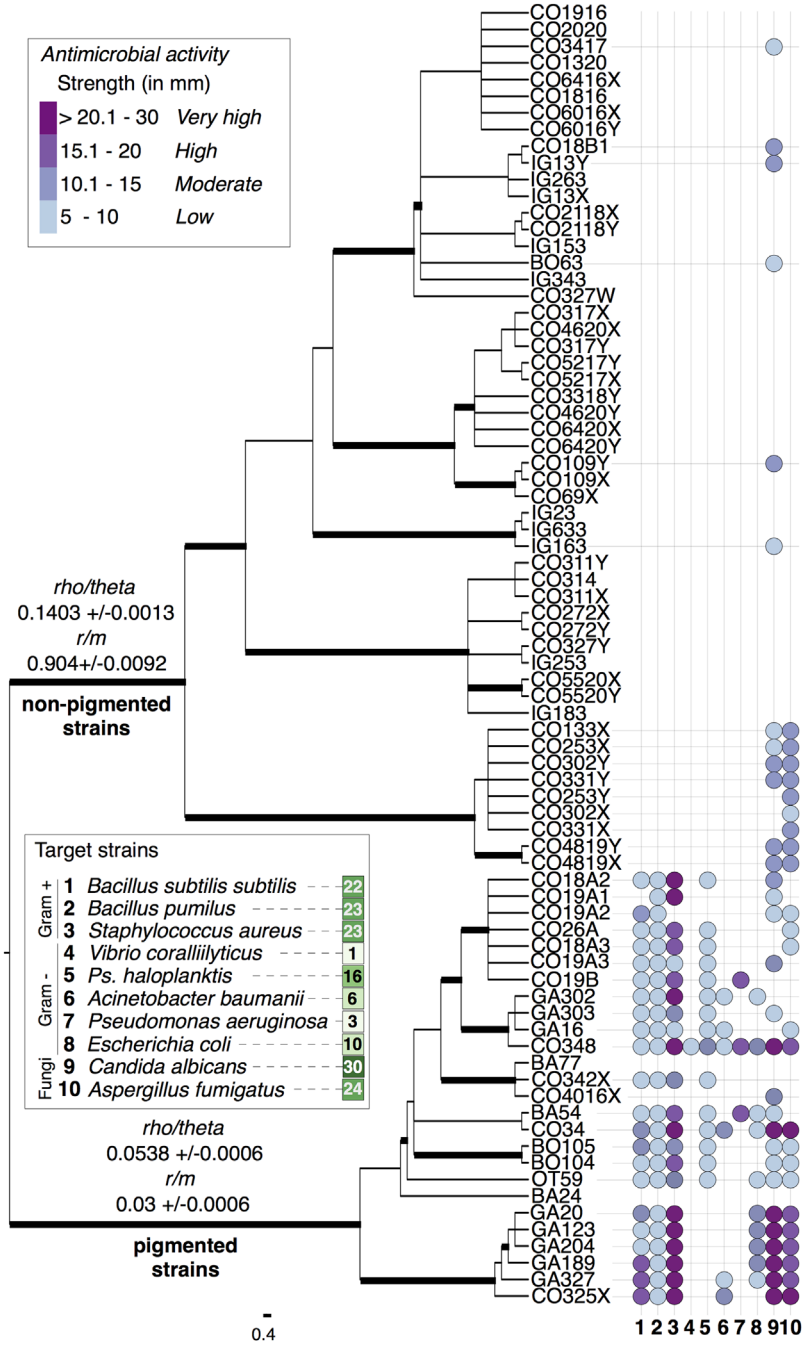

FIGURE 2 Consensus tree of *Pseudoalteromonas* strains from the marine environment of Panama. A 50% majority-rule consensus tree summarizing 3,003 trees of the posterior sample of three runs of ClonFrame v1.2 [24] based on 16S rRNA, *recA*, *rpoB* and *ftsZ* genes for 78 *Pseudoalteromonas* isolaaltes. Thickened branches indicate posterior probabilities >0.95. The *rho/theta* and *r/m* values are shown for each clade (non-pigmented and pigmented strains). The antimicrobial activity is tracked for each isolate using colored circles indicating their respective level of strength. A summary of the antimicrobial activity by target strain is shown in a square to the left of the figure

The non-pigmented clade included six highly supported clades, while only three highly supported clades were inferred for the pigmented clade (Fig. 2).

According to the CF analysis, recombination was less frequent than mutation in our dataset (*rho/theta* = 0.2407 +/− 0.0016). However, the impact of recombination on introducing variation was higher than that of mutation, being *r/m* = 2.3636+/− 0.0141 (for details see Fig. 2 and methods). When looking at the non-pigmented and pigmented clades separately, we found that recombination played a negligible role in the diversification of the pigmented strains (*rho/theta* = 0.0538+/− 0.0006, r/m = 0.03+/− 0.0006), while it is almost as important as mutation in the non-pigmented clade (*rho/theta* = 0.1404+/− 0.0013, *r/m* = 0.904+/− 0.0092).

The delta (δ) value in CF analyses shows the average track length of a recombination event [31]. In our study, the δ value for the non-pigmented clade was 96.5+/ − 1.0 bp, accounting for about 3.7% of the mean concatenated sequence length (2618 bp). For the pigmented clade the δ value was 1397.7 bp +/ − 15.76, representing ~53% of the mean concatenated sequence length for this group (2602 bp).

3.3 | Clonal diversity

CF analysis of the dataset composed of Panama strains and *Pseudoalteromonas* reference and type strains resulted in 43 clonal lineages. Of these, 23 belonged to the non-pigmented lineage (Fig. 3A), and 13 to the pigmented lineage (Fig. 3B). Seven clonal lineages were composed of only reference strains from GenBank (Fig. 3B). None of the non-pigmented clonal lineages from Panama grouped with reference strains (Fig. 3A). Conversely, four pigmented lineages matched with reference strains (Fig. 3B): lineage 30 (CO325X, GA327) grouped with *P. ruthenica* CP76, lineage 33 (BA24) grouped with *P. spongiae* UST010723-006, lineage 36 (CO34) grouped with *P. flavipulchra* JG1, and lineage 37 (BA54) grouped with *P. piscicida* ATCC 15057. The remaining nine pigmented lineages (31–32, 35, 38–43) from Panama were not related to known *Pseudoalteromonas* species in our analysis.

By aligning the 16S rRNA gene sequences of each clonal lineage against the high quality ribosomal RNA SILVA database we found that additional Panama lineages are related to reference *Pseudoalteromonas* strains (Fig. 3, Supporting Information Table S3). In particular, lineages 14 and 21–23 were closely related to the type strain *P. arabiensis* k53^T. No match was found for the remaining non-pigmented lineages (lineages 1–13 and 15–20). Within the pigmented lineages, the 31 and 32 are closely related to *P. ruthenica* (strains KMM290, KMM300, CP76 and clone JIV-49), and lineages 35, 40, and 42 are closely related to *P. piscicida* NBRC 103038. Surprisingly, the pigmented lineages 38–39, 41, and 43, grouped with *P. elyakovii* ATCC 700519, which is described as a non-pigmented species. However, the close relationship of the reference strain *P. elyakovii* NBRC 103035 to some of our non-pigmented lineages suggests a misidentification of one of the strains that belong this specie. The inconsistencies in species classification will likely be addressed when whole genome information for the investigated strains becomes available.

3.4 | LD analysis

By reconstructing the allelic profile of each isolate, we detected 40 out of 78 possible unique sequence types (STs, methods and Supporting Information Table S4). The clonal lineages 1, 6, 14, 22 (non-pigmented), and 41 (pigmented) were the most diverse, encompassing three STs, while other lineages were represented by a single STs. Association indices for each dataset are shown in Table 2. Significant linkage disequilibrium was detected in all datasets. There was no significant difference in the association index scores (I_A and rBarD) when they were calculated from the "population" or STs datasets for the whole dataset, as well as for the pigmented and non-pigmented datasets separately. The highest value was detected for the pigmented dataset (as both "population" and STs), suggesting that in this group there is higher linkage disequilibrium. In the non-pigmented group a clonal structure was also detected, but the association indices (I_A and rBarD) were lower, suggesting slightly weaker clonality. The two association indices showed a remarkable clonal structure suggesting that although recombination is present in the *Pseudoalteromonas* strains of Panama, it is not frequent enough to break the association among alleles.

3.5 | Antimicrobial activity

We tested the antimicrobial activity of *Pseudoalteromonas* strains from Panama (Fig. 2). Of the 78 strains tested, 39 strains showed antimicrobial activity against target bacteria and fungi. The yeast *Candida albicans* was the most susceptible target to *Pseudoalteromonas* strains (30 strains), followed by the fungus *Aspergillus fumigatus* (24 strains), and bacteria *Bacillus pumilus* (23 strains), *Staphylococcus aureus* (23 strains), and *Bacillus subtilis* (for details see Fig. 2 and Supporting Information Table S2). Bioactivity was mainly restricted to the pigmented clade in which the majority of the strains and all clonal lineages (Fig. 3B) displayed activity against Gram-positive bacteria. From this clade, the *Pseudoalteromonas* strain CO348, isolated from the mucus of *Leptogogia cofrini* (octocoral) displayed a broad range of bioactivity and was able to inhibit all the targets. All the remaining strains, isolated from sponges (*Amphimedon compressa* and *Niphates erecta*), in the same clade showed antimicrobial activity. Remarkably, 15 non-pigmented strains were active against the two fungal targets (Fig. 2). In this group, we found antifungal activity in a single monophyletic group composed of three clonal lineages (Fig. 3A) isolated from different species of octocorals (*Pacifigorgia smithsoniana*, *Psammogorgia* sp., *Pacifigorgia cairnsi* and *Leptogorgia tabogilla*) and an unidentified bryozoan collected in Coiba National Park (Fig. 1).

4 | DISCUSSION

In this study we analyzed the diversity and genetic structure of *Pseudoalteromonas* strains and showed that pigmented and non-pigmented strains are widely distributed in the marine

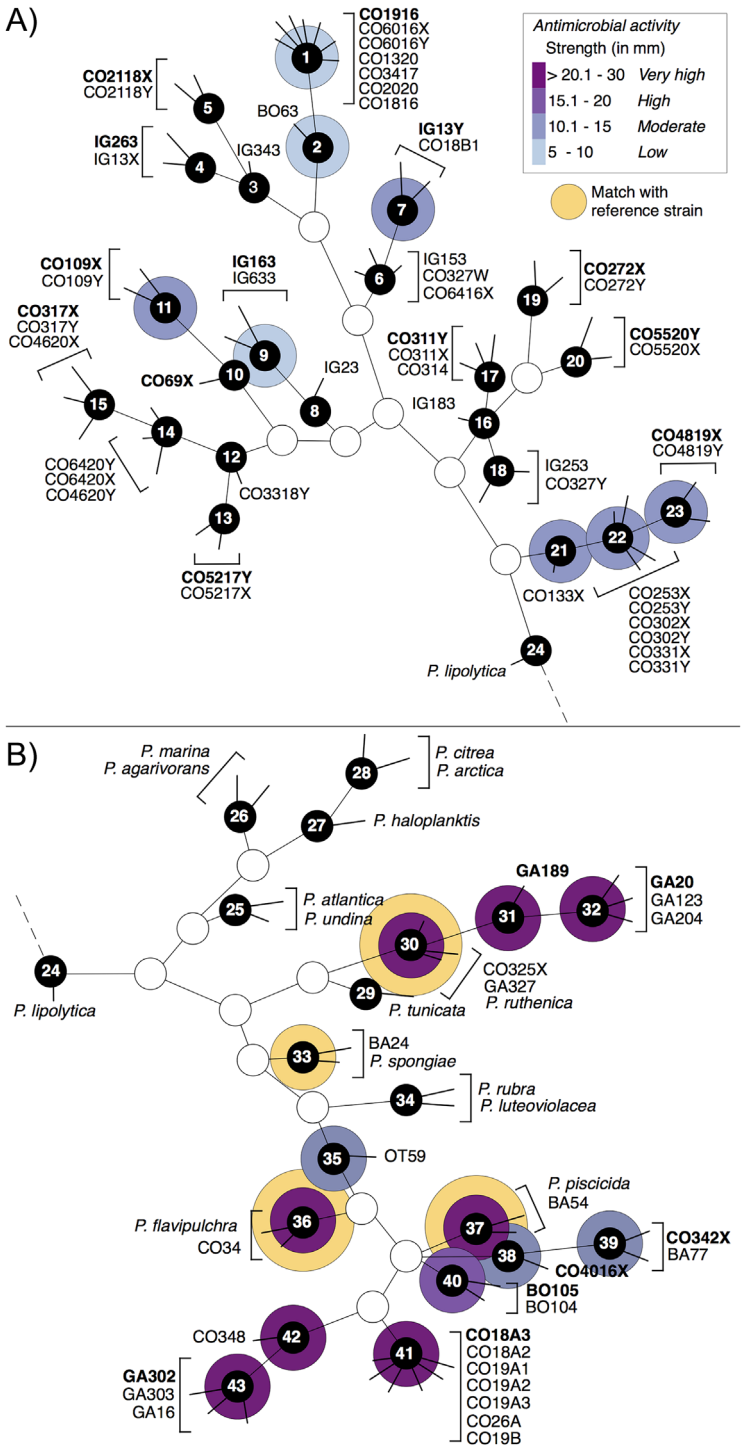

FIGURE 3 A network representation of clonal genealogy of *Pseudoalteromonas* strains from the marine environment of Panama. A) Non-pigmented lineages; B) pigmented lineages and reference strains used in this study. The network shows inferred clonal lineages (ancestral nodes) in black circles; each line near to the node indicates a single isolate. The strain considered as type for the each ancestral node is in bold. If the type of an ancestral node was not found among the isolates, it is shown as an empty circle. The antimicrobial activity strength is shown for each clonal lineage, from low (light blue) to very high (purple). Isolate matching with reference strains from GenBank is indicated with a yellow circle

environment of Panama. Furthermore, we identified strains of *Pseudoalteromonas* that inhibit the growth of selected microbial species, an activity that may be important for its host protection against pathogens.

By detecting *Pseudoalteromonas* strains using genus-specific PCR primers for 16S rRNA gene and sequencing of four loci we found that this genus has a wide host range, being found mainly associated with octocorals and stony corals in

TABLE 2 Association indices in *Pseudoalteromonas* of Panama derived with the program Multilocus v1.3b [30]

	Ia		rBarD		
	Population	**STs**	**Population**	**STs**	**n**
Whole data set	1.59382	1.02318	0.538596	0.358217	78
Pigmented	1.73198	1.30696	0.582168	0.439631	26
Non-pigmented	1.47235	0.931161	0.502025	0.329477	52

Association indices for the whole data set, for non-pigmented, and pigmented strains were calculated from two data sets, a "population dataset" including all strains. And a "STs dataset" in which identical sequences were collapsed into a single and distinct multilocus genotype. $*P < 0.001$.
n indicates the number of strains per sample analyzed.

Panama (mainly from Coiba National Park) as well as sponge, oysters, algae, sea urchins, and it can also occur in seawater. This finding is in agreement with published data on *Pseudoalteromonas* habitats, which include tight symbiotic associations with eukaryotic hosts as well as a free-living state [7,11]. In Panama, *Pseudoalteromonas* have been previously found in association with stony corals such as *Montastrea franksi* in Bocas del Toro [14], and octocorals like *Leptogorgia alba* from Isla Otoque [35,36].

We used a multilocus phylogenetic analysis taking into account recombination events to infer the genetic relationships of *Pseudoalteromonas* strains, and to assess whether particular clades are more likely to possess antimicrobial activity. This multilocus phylogenetic analysis was chosen because the analysis based solely on the 16S rRNA gene lacks resolving power to clearly differentiate species [37], as shown by the lack of backbone support on most clades in the present 16S rRNA gene phylogeny (Fig. 1). An example of a poorly resolved group consists of the *Pseudoalteromonas* strains from Panama (GA123, GA204, GA327, GA189 y CO325X), which are closely related to the pale-orange-pigmented species *P. ruthenica* (KMM300^T and CP76). This clade was included with low bootstrap support in the non-pigmented clade according to 16S rRNA gene phylogeny (Fig. 1), but it was placed with high confidence in the pigmented group in the concatenated 4-locus phylogeny. The addition of housekeeping genes, such as *recA*, *rpoB* and *ftsZ* provides higher support in phylogenetic estimations, as our results suggest [38]. We found a high clonal structure of *Pseudoalteromonas* in the Panamanian marine environments where, many of the inferred clonal lineages displayed high bioactive potential.

To date there have been few reports on the bioactivity of non-pigmented strains of *Pseudoalteromonas*. Interestingly, our results (Fig. 2) showed that in the non-pigmented clade some strains of Panamanian *Pseudoalteromonas* have antifungal activity. This activity is concentrated in three clonal lineages and includes strains mostly isolated from octocorals (Fig. 3, Table 1). This group is closely related to *P. arabiensis*, which is known to produce exopolysaccharides, rather than bioactive small molecules against fungi [39].

The high bioactivity previously reported for pigmented strains [7] is confirmed by our results. We found that several pigmented strains show a range of antimicrobial activity against bacteria and fungi, and some of our strains are very closely related to bioactive reference strains. For example, the strains grouped in the lineages 30–32 (Fig. 3B) isolated from sponges and octocorals from Punta Galeta and Coiba National Park are closely related to *P. ruthenica*, a species previously isolated from saltern environments and mussels, with antimicrobial activity against several marine bacteria [40]. Studies have reported the production of haloprotease CPI in this species, suggesting that it aids the species in coping with extreme habitat conditions [41]. Four pigmented clonal lineages (35, 37, 40, and 42) are closely related to the opportunistic fish pathogen *Pseudoalteromonas piscicida*. This species possesses antibacterial activity and produces the compound norharman, known to have cytotoxic activity toward cervical and stomach cancer [42]. Our strains were found in association with different species of octocorals from Isla Otoque, Bocas del Toro and Coiba National Park and, similar to *P. piscicida*, possess high antimicrobial and antifungal activity. Strain CO34 is highly similar to the golden yellow colored *P. flavipulchra*, which synthesizes a protein (PfaP) and also small molecules that showed great antibacterial activities against fish pathogens and have the potential to be used as a probiotic or antibiotic in aquaculture [43].

The antimicrobial activity showed by Panamanian *Pseudoalteromonas* strains, mainly isolated from octocorals (mucus layer and tissue), suggests that the presence of coral-associated bacteria could help the coral to avoid pathogen invasion and contribute to infectious disease resistance [36].

Despite our findings of antifungal activity on the non-pigmented clade, our results are in agreement with the general observation that pigmented strains are more bioactive.

Overall, *Pseudoalteromonas* strains from Panama showed a highly clonal structure despite the strong recombination effect found in the non-pigmented clade. This is different to the trend that has been reported for aquatic and marine bacteria such as *Vibrio vulnificus*, *Microcystis aeruginosa*, and *Plesiomonas shigelloides* where high rates of

recombination are the norm [44]. By contrast, in the pigmented clade recombination effects were found to be negligible. At the individual population level, a deeper sampling with more replicates per clonal lineage is required to confirm the present trend. The found trend of highly clonal structure could be due to the biology of most of the *Pseudoalteromonas* strains analyzed: symbiotic associates of marine invertebrates, in which the maintenance of genotypic uniformity of an associated microorganism within the host could promote a long-term and stable mutualistic symbiosis. It will be of interest to explore the relationships between the genetic structure and transmission mode (vertical vs. horizontal) of *Pseudoalteromonas* species to see how this compares to existing theories of host-microbe mutualisms [45].

The lower average track length of a recombination event (delta value) suggests that mobile elements may be present in the non-pigmented clade [31]. The effect of recombination in the non-pigmented clade might be related to potential drivers of recombination (e.g., bacteriophages and plasmids), and may be the reason behind the higher diversity in this clade. In other words, the activity of mobile elements is a possible mechanism promoting the observed effect of recombination in the non-pigmented clade of *Pseudoalteromonas* that deserves to be explored. Bacteriophages and plasmids have been previously reported in non-pigmented and pigmented *Pseudoalteromonas* strains. More than 70% (8/11) of *Pseudoalteromonas* strains which both, presence or absence of pigmentation and presence of mobile elements, reported in the scientific literature, are non-pigmented strains (see Supporting Information Table S11). Examples of mobile elements in *Pseudoalteromonas* includes ϕRIO phage in *P. marina* mano4 [46], B8b phage in *Pseudoalteromonas* sp. QC-44 [47], phage PH357 in *P. lipolytica* BH357 [48] and the plasmids pMBL6842 [49] and pSM429 [50] harbored by *P. rubra* SCSIO 6842 and *Pseudoalteromonas* sp. BSi20429 respectively (Supporting Information Table S11). These studies stress the possibility that mobile elements play an important role driving microbial genetic exchange in the genus [47,50]. Similarly, mobile elements may be involved in horizontal gene transfer, and provide an adaptive advantage for the bacterial host in the marine environment. It cannot be excluded that these mobile elements influence the degree of recombination found in the genus.

Pseudoalteromonas is widely distributed in the marine environments of Panama, being often found in association with marine organisms, although they can also be found as free-living cells. Most of the Panamanian *Pseudoalteromonas* strains analyzed did not group with reference and type strains. As most of the reference and type strains are from temperate regions, the results suggest that the diversity of *Pseudoalteromonas* may be potentially higher in tropical environments. Our results show that there are several putative new species of *Pseudoalteromonas* in Panama to be described. Moreover our results suggest that the presence of these microorganisms associated to marine invertebrates may be important for protecting against pathogens via the production of secondary metabolites [7,14]. A clonal structure where recombinational replacements are present, as was found in this study, could represent an advantage in the marine environment. The reason is because it could allow the diversification of genotypes for host colonization, nutrient acquisition, production of enzymes and secondary metabolites necessary for the strain's success in their habitat [15].

Whole-genome sequence information for the investigated *Pseudoalteromonas* strains will also allow for the identification of candidate biosynthetic gene clusters that could eventually be linked to bioactive natural products. The study of the microbiota associated with the marine environment, their interactions and its potential as antimicrobials producers will help to confirm the importance of the preservation of these landscapes in Panama, which are threatened by harmful biotic and abiotic factors.

In summary, we found evidence that species of *Pseudoalteromonas* that occurs in the tropical marine environment of Panama have a highly clonal structure, an exception to what has been generally found for aquatic and marine bacteria. Overall, mutation is more frequent than recombination in *Pseudoalteromonas*, but this varies between pigmented and non-pigmented clades. Interestingly, several putative new species of *Pseudoalteromonas* to be described has been uncovered using the ClonalFrame analysis, displaying a higher diversity of this genus in tropical environments. Finally, antimicrobial activity on pigmented and non-pigmented strains suggest that this group of microorganisms could play an important role in their host defense against pathogens by the production of secondary metabolites with high potential for marine natural products drug discovery.

ACKNOWLEDGMENTS

We gratefully acknowledge the Government of Panama (Ministerio de Ambiente) for granting permission to collect the marine organisms used in this study. This work was partially supported by the National Secretariat for Science and Technology of Panama (SENACYT, grant numbers COL08-061 and COL09-047), the Fogarty International Center's International Cooperative Biodiversity Groups program (grant number TW006634), and the Global Environmental Fund (GEF ID 4780–UNDP 81860), the Smithsonian Tropical Research Institute, and the research funding program Landes-Offensive zur Entwicklung Wissenschaftlich-Ökonomischer Exzellenz (LOEWE) of Hessen State Ministry for Higher Education, Research, and the Arts through the Senckenberg Biodiversity and Climate Research Centre

(BiK-F). The authors thank to Javier Ballesteros, Joel Sánchez and Carlos Guevara for their work in the collection of marine organisms as well as in the isolation process, cryopreservation and characterization of marine bacterial strains and Odalisca Breedy for confirming the species of soft corals. LCM received support from SENACYT SNI program.

CONFLICTS OF INTEREST

The authors indicate no conflicts of interest.

ORCID

Luis C. Mejía
Marcelino Gutiérrez http://orcid.org/0000-0003-1423-3772

REFERENCES

[1] Blunt JW, Copp BR, Keysers RA, Munro MHG. Marine natural products. Nat Prod Rep 2016;33:382–431.
[2] Bourguet-Kondracki ML, Kornprobst JM. Promising marine molecules in pharmacology, In: La Barre S, Kornprobst JM, editors. Outstanding marine molecules: chemistry, biology, analysis, Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA; 2014. pp 245–64.
[3] Piel J. Metabolites from symbiotic bacteria. Nat Prod Rep 2009;26:338–62.
[4] Gerwick WH, Fenner AM. Drug discovery from marine microbes. Microb Ecol 2013;65:800–6.
[5] Debbab A, Aly AH, Lin WH, Proksch P. Bioactive compounds from marine bacteria and fungi. Microb Biotechnol 2010;3:544–63.
[6] Zheng L, Han X, Chen H, Lin W. Marine bacteria associated with marine macroorganisms: the potential antimicrobial resources. Ann Microbiol 2005;55:119–24.
[7] Bowman JP. Bioactive compound synthetic capacity and ecological significance of marine bacterial genus *Pseudoalteromonas*. Mar Drugs 2007;5:220–41.
[8] Ivanova EP, Flavier S, Christen R. Phylogenetic relationships among marine *Alteromonas*-like proteobacteria: emended description of the family *Alteromonadaceae* and proposal of *Pseudoalteromonadaceae* fam. nov., *Colwelliaceae* fam. nov., *Shewanellaceae* fam. nov., *Moritellaceae* fam. nov., *Ferrimonadaceae* fam. nov., *Idiomarinaceae* fam. nov. and *Psychromonadaceae*. Int J Syst Evol Microbiol 2004;54:1773–88.
[9] Skovhus TL, Ramsing NB, Holmström C, Kjelleberg S. Real-Time Quantitative PCR for assessment of abundance of *Pseudoalteromonas* Species in marine samples. Appl Environ Microbiol 2004; 70:2373–82.
[10] Gauthier G, Gauthier M, Christen R. Phylogenetic analysis of the genera *Alteromonas*, *Shewanella*, and *Moritella* using genes coding for small-subunit rRNA sequences and division of the genus *Alteromonas* into two genera, *Alteromonas* (emended) and *Pseudoalteromonas* gen. nov., and proposal of twelve new species combinations. Int J Syst Bacteriol 1995;45:755–61.
[11] Ivanova EP, Ng HJ, Webb HK. In: Rosenberg E, De Long EF, Lory S, Stackebrandt E, editors. The Family Pseudoalteromonadaceae, Berlin: Springer Berlin Heidelberg; 2014. pp 575–82.
[12] Bian F, Xie BB, Qin QL, Shu YL. Genome sequences of six *Pseudoalteromonas* strains isolated from Arctic Sea ice. J Bacteriol 2012;194:908–9.
[13] Roberts CM, McClean CJ, Veron JEN, Hawkins JP. Marine biodiversity hotspots and conservation priorities for tropical reefs. Science 2002;295:1280–4.
[14] Rohwer F, Seguritan V, Azam F, Knowlton N. Diversity and distribution of coral-associated bacteria. Mar Ecol Prog Ser 2002;243:1–0.
[15] Spratt BG, Woodford N, Johnson AP. Exploring the concept of clonality in bacteria, Genomics, Proteomics, and Clinical Bacteriology: Methods in Molecular Biology, Totowa, New Jersey: Homana Press; 2004. pp 323–52.
[16] Jensen PR, Gontang E, Mafnas C, Mincer TJ. Culturable marine actinomycete diversity from tropical Pacific Ocean sediments. Environ Microbiol 2005;7:1039–48.
[17] Blanco-Abad V, Ansede-Bermejo J, Rodriguez-Castro A, Martinez-Urtaza J. Evaluation of different procedures for the optimized detection of *Vibrio parahaemolyticus* in mussels and environmental samples. Int J Food Microbiol 2009;129:229–36.
[18] Frank JA, Reich CI, Sharma S, Weisbaum JS. Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. Appl Environ Microbiol 2008;74:2461–70.
[19] Contreras-Moreira B, Sachman-Ruiz B, Figueroa-Palacios I, Vinuesa P. Primers4clades: a web server that uses phylogenetic trees to design lineage-specific PCR primers for metagenomic and diversity studies. Nucleic Acids Res 2009;37:W95–100.
[20] Lane D, Stackebrandt E, Goodfellow M. 16S/23S rRNA sequencing, Nucleic Acids Techniques in Bacterial Systematics, Weinheim: John Wiley & Sons, Chichester; 1991. pp 115–47.
[21] Wang Q, Garrity GM, Tiedje JM, Cole JR. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 2007;73:5261–7.
[22] Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol 2013;30:772–80.
[23] Stamatakis A. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 2006;22:2688–90.
[24] Didelot X, Falush D. Inference of bacterial microevolution using multilocus sequence data. Genetics 2007;175:1251–66.
[25] Didelot X, Lawson D, Darling A, Falush D. Inference of homologous recombination in bacteria using whole-genome sequences. Genetics 2010;186:1435–49.
[26] López-Pérez M, Gonzaga A, Rodriguez-Valera F. Genomic diversity of "deep ecotype" *Alteromonas macleodii* isolates: evidence for pan-mediterranean clonal frames. Genome Biol Evol 2013;5:1220–32.
[27] Gelman A, Rubin D. Inference from iterative simulation using multiple sequences. Stat Sci 1992;7:457–511.
[28] Pruesse E, Peplies J, Glöckner FO. SINA: accurate high-throughput multiple sequence alignment of ribosomal RNA genes. Bioinformatics 2012;28:1823–9.

[29] Smith JM. Estimating the minimum rate of genetic transformation in bacteria. J Evol Biol 1994;7:525–34.
[30] Agapow PM, Burt A. Indices of multilocus linkage disequilibrium. Mol Ecol Notes 2001;1:101–2.
[31] D'Alelio D, Salmaso N, Gandolfi A. Frequent recombination shapes the epidemic population structure of *Planktothrix* (Cyanoprokaryota) in Italian subalpine lakes. J Phycol 2013;49:1107–17.
[32] Smith JM, Smith NH, O'Rourke M, Spratt BG. How clonal are bacteria?. Proc Natl Acad Sci USA 1993;90:4384–8.
[33] Castillo I, Lodeiros C, Núñez M, Campos I. Evaluación in vitro de sustancias antibacterianas producidas por bacterias aisladas de diferentes organismos marinos. Rev Biol Trop 2001; 49:1213–22.
[34] Rypien KL, Ward JR, Azam F. Antagonistic interactions among coral-associated bacteria. Environ Microbiol 2010;12:28–39.
[35] Nguyen DD, Wu CH, Moree WJ, Lamsa A. MS/MS networking guided analysis of molecule and gene cluster families. Proc Natl Acad Sci USA 2013;110:E2611–20.
[36] Moree WJ, McConnell OJ, Nguyen DD, Sanchez LM. Microbiota of healthy corals are active against fungi in a light-dependent manner. ACS Chem Biol 2014;9:2300–8.
[37] Tindall BJ, Rosselló-Móra R, Busse HJ, Ludwig W. Notes on the characterization of prokaryote strains for taxonomic purposes. Int J Syst Evol Microbiol 2010;60:249–66.
[38] Vynne NG, Mansson M, Gram L. Gene sequence based clustering assists in dereplication of *Pseudoalteromonas luteoviolacea* strains with identical inhibitory activity and antibiotic production. Mar Drugs 2012;10:1729–40.
[39] Matsuyama H, Minami H, Kasahara H, Kato Y. *Pseudoalteromonas arabiensis* sp. nov., a marine polysaccharide-producing bacterium. Int J Syst Evol Microbiol 2013;63:1805–9.
[40] Kalinovskaya NI, Ivanova EP, Alexeeva YV, Gorshkova NM. Low-molecular-weight, biologically active compounds from marine *Pseudoalteromonas* species. Curr Microbiol 2004;48: 441–6.
[41] de la Haba RR, Sánchez-Porro C, León MJ, Papke RT. Draft genome sequence of the moderately halophilic bacterium *Pseudoalteromonas ruthenica* strain CP76. Genome Announc 2013;1:e00268–13.
[42] Zheng L, Yan X, Han X, Chen H. Identification of norharman as the cytotoxic compound produced by the sponge (Hymeniacidon perleve)-associated marine bacterium Pseudoalteromonas piscicida and its apoptotic effect on cancer cells. Biotechnol Appl Biochem 2006;44:135–42.
[43] Yu M, Tang K, Liu J, Shi X. Genome analysis of *Pseudoalteromonas flavipulchra* JG1 reveals various survival advantages in marine environment. BMC Genomics 2013;14:707.
[44] Vos M, Didelot X. A comparison of homologous recombination rates in bacteria and archaea. ISME J 2009;3:199–208.
[45] Hernandez-Agreda A, Gates RD, Ainsworth TD. Defining the core microbiome in corals' microbial soup. Trends Microbiol 2017; 25:125–40.
[46] Hardies SC, Hwang YJ, Hwang CY, Jang GI. Morphology, physiological characteristics, and complete sequence of marine bacteriophage φRIO-1 infecting *Pseudoalteromonas marina*. J Virol 2013;87:9189–98.
[47] Lara E, Holmfeldt K, Solonenko N, Sà EL. Life-Style and genome structure of marine Pseodoalteromonas Siphovirus B8b isolated from the Northwestern Mediterranean sea. PloS ONE 2015;10: e114829.
[48] Gong Z, Wang M, Yang Q, Li Z. Isolation and complete genome sequence of a novel *Pseudoalteromonas* phage PH357 from the Yangtze River Estuary. Curr Microbiol 2017;74:832–9.
[49] Li B, Wang P, Zeng Z, Cai X. Complete genome sequence of *Pseudoalteromonas rubra* SCSIO 6842, harboring a putative conjugative plasmid pMBL6842. J Biotechnol 2016;224:66–7.
[50] hao ZD, Yu Z, Li P, Wu Z. Characterization of cryptic plasmid pSM429 and its application for heterologous expression in psychrophilic *Pseudoalteromonas*. Microb Cell Fact 2011; 10:30.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Atencio LA, Dal Grande F, Young GO, et al. Antimicrobial-producing *Pseudoalteromonas* from the marine environment of Panama shows a high phylogenetic diversity and clonal structure. *J Basic Microbiol*. 2018;58: 747–769. https://doi.org/10.1002/jobm.201800087