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ORIGINAL PAPER

Assessment of Plant-Growth Promoting Potential of Bacteria Isolated from Amazonian Black Pepper Roots

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Abstract
Purpose: In the Amazon, farmers use chemical fertilizers on a large scale to meet the nutritional requirements of some crops. Plant-growth promoting bacteria (PGPB) offer a sustainable alternative to enhance crop productivity. This study aimed to prospect novel PGPB from Amazonian black pepper (*Piper nigrum* L.) roots. Methods: Bacterial isolates were obtained from plant roots, evaluated for their biofertilizing potential, and the most promising strain was selected for genome sequencing. Taxonomic classification was based on 16 S rRNA gene sequencing. ACC deaminase activity, phosphate solubilization, and nitrogen fixation were assessed. Genome sequencing was performed using the Ion GeneStudio S5 platform. Results: The 20 isolates were affiliated to *Enterobacter* (7 isolates), *Klebsiella* (4 isolates), *Kosakonia* (5 isolates), *Bacillus* (2 isolates), and two unclassified bacteria. Seven isolates were positive for ACC deaminase activity, while four were positive for the presence of the *nifH* gene. Nitrogenase gene was found only in *Kosakonia* isolates. Ninety isolates were able to solubilize phosphate. The isolate Pn16 was the most promising and presented a genome of 6,432,985 bp, GC content of 55%, 6,465 Coding Sequences, 10 Symbiotic Islands, 28 biosynthetic gene clusters, and several genes involved in plant-growth promotion such as *phoU-pstSCAB-phoBR*, *oqxAB*, *ipdC*, *speADEGF, nifHDK*. Conclusions: We were able to isolate a bacterium with potential for biofertilization. Based on phylogeny and Average Nucleotide Identity, we propose the classification of the Pn16 isolate as *Kosakonia pseudosacchari* Pn16.

Keywords Plant-growth Promoting bacteria · *Piper nigrum* L. · *Kosakonia Pseudosacchari* · Prospecting · Biocontrol · *nifH*

1 Introduction

Endophytic bacteria able to promote plant growth are a promising source for the development of products for sustainable agriculture (Chouhan et al. 2021). Despite the old and extensive studies and the long list of commercialized bacterial inoculants (Glick 2012), the use of biofertilizers in the Amazon region is scarce (Oliveira et al. 2020). These bacteria help plants to grow through direct and/or indirect processes such as biological nitrogen fixation, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity, and phosphate solubilization (Jeon et al. 2003; Marques et al. 2010; Mendes et al. 2018; Pereira et al. 2019; Santos et al. 2021). Moreover, strains of Plant Growth-Promoting Bacteria (PGPB) have been isolated from several environments and exert effects on a range of crop species (Katsenios et al. 2022). Studies have revealed that endophytes from the same plant species may have developed specific adaptations to the environmental conditions of the host plant, such as soil type, climate, and disease pressures., and can be more efficient and competitive compared to the non-indigenous strains (Verma et al. 2013). In a recent study, Nascimento et al. 2021 quantified the effect of inoculation of native diazotrophic bacteria on seedlings of *Eucalyptus uruphylla.*

PGPB have been used as biostimulants (Lopes et al. 2021), biofertilizers (Cortivo et al. 2017), and for biocontrol

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(Nascimento et al. 2015; Toh et al. 2016). Kodithuwakku and colleagues (2016) evaluated different combinations of inoculants for black pepper growth and demonstrated that plants inoculated with *Trichoderma sp*. and/or *Pseudomonas fluorescens* exhibited higher leaf area, shoot height, root volume, and shoot and root dry mass compared to the control group. PGPBs have been isolated from black pepper (Oliveira et al. 2020), brachiaria (Hungria et al. 2021), and lettuce (Cardoso et al. 2019), among other plants. Some microbial species that have shown potential for the promotion of black pepper growth were the rhizobacterium *Bacillus velezensis* RB.DS29 (Trinh et al. 2019), the endophytic bacteria *Klebsiella* sp. and *Enterobacter* sp. (Jasim et al. 2013), and the fungus *Piriformospora indica* (Anith et al. 2018). Black pepper (*Piper nigrum* L.) belongs to Piperaceae family, and it consists of one of the most important agricultural crops in the world. The Singapore cultivar started to be commercially exploited around 1933 in Tomé-Açu, Pará, Brazilian Amazon (Lemos, 2011). The state of Pará produces around 36,156 tons of black pepper, representing 32% of Brazilian production (IBGE, 2020). Thus, due to its socioeconomic importance it becomes a promising source for prospecting biotechnological products such as PGPB.

Among the PGPB described so far, *Kosakonia* spp. have demonstrated excellent results for use as biofertilizer (Quintas-Nunes et al. 2022). Genomic data have provided valuable insights into the genetic diversity, evolutionary adaptation and biotechnological applications of this taxon. Recombination mechanisms and gene acquisition were identified as significant drivers of genome evolution in *Kosakonia* (Jan-Roblero et al. 2020; Quintas-Nunes et al. 2022). They have been described in several ecological niches, from human tissues to plants (Mertschnigg et al. 2020; Yang, 2018). The genome of *Kosakonia radicincitans* strain MUSA4, a diazotrophic bacterium isolated from banana leaves, presented several characteristics to promote plant growth including phosphate solubilization, nitrogen fixation, and the production of indole acetic acid, siderophores, acetone, and polyamine (Quintas-Nunes et al. 2022). In contrast, Zhang and colleagues (2022) described a phytopathogenic strain *Kosakonia* sp. Pa82 infecting Patchouli plants in Guangdong, China. Genes involved in virulence, adhesion, biofilm formation, and endotoxin were predicted on its genome. These findings highlight the metabolic and ecological diversity of the genus and the importance of additional studies.

The use of PGPBs contributes to sustainable agriculture by reducing the application of chemical fertilizers in the soil. Thus, the aim of this study was to isolate endophytic bacteria from a *P. nigrum* crop at the Brazilian Amazonia and evaluate their capacity to promote plant growth through molecular e microbiological methods. Additionally, the genome of the isolate with the highest potential for biological fertilization was sequenced on the Ion GeneStudio S5 platform.

2 Materials and Methods

2.1 Bacterial Isolation

Root samples of black pepper (*P. nigrum* L. cultivar Bragantina) were collected at the Baião city, state of Pará, Brazil, in April 2019 (02º47'26'' S and 49º40'18'' W). Samples were stored in ~ 10ºC and processed within 4 h after collection. Endophytic bacteria were isolated, according to Fidalgo and colleagues (2016). Roots (2 to 5 g) were cleaned with Phosphate-Buffered Saline (PBS) 1X to remove the soil. Samples were immersed in ethanol 96% for 1 min, sodium hypochlorite 5% for 30 min, ethanol 96% for 1 min, and washed with sterile distilled water three times. Tissues were macerated with a mortar and pestle for endophytic bacteria isolation. A serial dilution in saline solution (NaCl 0.95%) was performed, and aliquots of 100 µL from dilutions 10^{-4} to 10^{-8} were plated on TSA supplemented with cycloheximide 100 µg mL^{-1}. Plates were incubated for up to 72 h at 28 ± 2 °C. Bacterial colonies with different morphologies were selected, and axenic cultures were obtained by the streak plate method. All colonies that presented different characteristics were isolated (colony shape and color). The technical and financial capacity of the project was also taken into consideration to define the number of total isolates. Isolates were Gram stained, and their morphology was visualized in the optic microscope Eclipse 80i (Nikon) coupled to a Ds-Ri1 camera (Nikon).

2.2 DNA Extraction and Taxonomic Classification

DNA extraction was performed using the phenol/chloroform/isoamyl alcohol method (Sambrook, 1989), and the DNA was quantified in nano spectrophotometer NanoDrop (Thermo Fisher Scientific). The 16S rRNA gene was amplified using primers 8F (5'-AGAGTTTGATCCTGGC TCAG-3') and 1492R (5'-GGTTACCTTGTTACGACT T-3'). The reaction was made in a final volume of 25 µL, containing 0.5 to 10 ng of template DNA, 1 µM of each primer; dNTPs 0.2 mM; $MgCl_2$ 2.5 mM, and 2.5 U of Taq DNA polymerase (Invitrogen). Cycling was performed in the GeneAmp 9700 (Thermo Fisher Scientific) with an initial step of denaturation of 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, and a final step of 72 °C for 10 min. The amplicons were sequenced in the ABI 3500 platform (Thermo Fisher Scientific). Forward and reverse sequences were compared

to the 16 S rRNA database from GenBank using BLASTn. Isolates Pn6, Pn11, Pn13, and Pn17 were sequenced only once (forward or reverse).

2.3 ACC Deaminase Activity Assay

Isolates were evaluated for 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity according to Penrose and Glick (2003) using DF minimum medium. Briefly, isolates were grown overnight in Tryptic Soy Broth (TSB), subsequently centrifuged at 10,000 g for 5 min and washed with PBS 1X three times to eliminate TSB. Isolates were inoculated in DF minimum medium supplemented with 3 mM of ACC (test group) as the sole source of nitrogen, without ACC (negative control), or with 2 g L^{-1} of $(NH_4)_2SO_4$ (positive control). Plates were incubated for up to 72 h at 28 ± 2 °C, and bacterial growth was checked daily.

2.4 Molecular Detection of nifH Gene by PCR

The ability to fix nitrogen was evaluated by the partial amplification of the *nifH* gene. PCR was performed using primers IGK3 (5'-GCIWTHTAYGGIAARGGIGGIATHG-GIAA-3') and DVV (5'-ATIGCRAAICCICCRCAIA-CIACRTC-3'). These primers were identified by Gaby and Buckley (2012) as those with the best performance for the amplification of *nifH* in a wide range of taxa. Reactions were made in a final volume of 25 µL, containing 1 µL of resuspended bacterial colonies, 1 µM of each primer, dNTPs 0.2 mM, $MgCl_2$ 2.5 mM and 2.5 U of Taq DNA polymerase (Invitrogen). All reactions were made in triplicate and a negative control was added. Thermal cycling was performed on GeneAmp 9700 system (Thermo Fisher Scientific) with an initial step of denaturation of 94 °C for 10 min, followed by 25 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and a final step of 72 °C for 10 min. Amplicons were visualized in 1% agarose gel stained with ethidium bromide. Isolates were positive when 383 bp fragments were visualized in all triplicates.

2.5 Phosphate Solubilization Assay

Phosphate solubilization was determined by growing the isolates in the NBRIP medium as described by Nautiyal (1999). Briefly, isolates were grown overnight in TSB, subsequently centrifuged at 10,000 g for 5 min and washed with PBS 1X three times to eliminate TSB medium. Cell density was adjusted to 0.5 McFarland. Isolates were inoculated in NBRIP medium by dropping 10 µL of the washed cultures in the solid medium in triplicate. Plates were incubated at 30 ± 2ºC for up to 7 days. The diameter of the transparent halo formed around the bacterial colony was measured and the result was expressed as solubilization efficiency (E) using the following formula proposed by Nguyen and colleagues (1992):

$$E = \frac{Solubilizationdiameter}{Growthdiameter} \times 100$$

Replicates were compared by Analysis of Variance (ANOVA) and the averages were compared using the Tukey test ($p < 0{,}05$).

2.6 Whole Genome Sequencing

The most promising isolate for plant growth promotion was selected for whole genome sequencing. DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's protocol. Nucleic acid was quantified in nano spectrophotometer NanoDrop (Thermo Fisher Scientific). Sequencing was performed in the Ion GeneStudio S5 platform (Thermo Fisher Scientific) using a fragment library that was prepared by the Ion Chef instrument (Thermo Fisher Scientific). The reads of up to 200 bp were trimmed and filtered using a cut-off Phred > 20 and a minimum size of 100 bp using Trimmomatic v.0.35 (Bolger et al. 2014). Assembly was performed using Velvet v.1.2.10 (Zerbino and Birney 2008) and a sequence scaffold was obtained using *Kosakonia pseudosacchari* BDA-62-3 as a reference genome in the software Contiguator v.2 (Galardini et al. 2011).

2.7 Comparative Genomics

Open Reading Frames (ORFs) were automatically predicted with Prokka v.1.14.15 (Seemann 2014). To confirm and improve the automatic annotation, BlastKOALA and KEGG were used (Kanehisa et al. 2016). Symbiotic Islands (SIs) were predicted using GIPSy v.1.1.2 (Soares et al. 2016) with default parameters. Biosynthetic gene clusters (BGCs) were predicted using AntiSMASH v.4.0 (Blin et al. 2017). Resistance genes were predicted using ResFinder v.4.0 (Bortolaia et al. 2020) and plasmids were predicted using PlasmidFinder v.2.0.1 (Carattoli and Hasman 2020). Fifteen *Kosakonia* complete genomes were downloaded from GenBank and used to calculate the pangenome of the genus with the Roary pipeline v.3.11.2 (Page et al. 2015). The genome sequence data was uploaded to the Type (Strain) Genome Server (TYGS), for phylogenomic analysis using standard parameters (Meier-Kolthoff and Göker 2019). Circular genome image was generated with BRIG v.0.95 (Alikhan et al. 2011). For gene content comparisons, reference genomes downloaded from GenBank were annotated with Prokka to normalize the predicted coding sequences.

Pairwise genome comparisons were performed using Average Nucleotide Identity (ANI) analysis in the online tool JSpeciesWS (Richter et al. 2015).

3 Results

3.1 Bacterial Isolation and Assessment of Plant-growth Promotion Characteristics

Twenty endophytic isolates were obtained from the black pepper roots. Eighteen strains were Gram-negative and two were Gram-positive. According to 16 S rRNA gene sequencing, they were affiliated to four genera: *Enterobacter* (7 isolates), *Kosakonia* (5 isolates), *Klebsiella* (4 isolates), *Bacillus* (2 isolates), and two unclassified bacteria. Identity values ranged from 98.80 to 100% (Table 1).

Seven isolates were positive for the ACC deaminase activity (Table 1) (Pn2, Pn3, Pn12, Pn13, Pn16, Pn17, and Pn20) and were affiliated to genera *Enterobacter*, *Klebsiella*, *Kosakonia*, and one unclassified bacterium (Table 1). Four isolates were positive for the *nifH* gene (Pn12, Pn16, Pn17, and Pn19). They were affiliated to the *Kosakonia* genus (Table 1). Finally, only one isolate was not capable of solubilizing inorganic phosphate. The isolates Pn17 and Pn10 presented the highest solubilization efficiency: 166% and 155%, respectively (Table 1). The isolate Pn13 presented the lowest solubilization efficiency, 19% (Table 1). Isolates with solubilization efficiency higher than 100% were detected in all genera: *Klebsiella*, *Bacillus*, *Enterobacter*, and *Kosakonia*.

3.2 Genomics of Kosakonia Pseudosacchari Pn16

Kosakonia pseudosacchari Pn16 presented a genome of 6,432,985 bp in size, GC content of 55%, 6,465 Coding Sequences (CDSs), and no plasmids were found (Fig. 1). The final scaffold was composed of 129 contigs with a N50 of 10,935 bp. Functional annotation identified 532 CDSs related to carbohydrates metabolism, 515 related to amino acid metabolism, 248 related to protein metabolism, and 246 related to cofactors, vitamins, and prosthetic groups. Ten Symbiotic Islands were detected by GIPSy and 28 potential Biosynthetic Gene Clusters (BGCs) were predicted by

Table 1 Taxonomic affiliation, BLAST identity and coverage, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity, *nifH* detection, and phosphate solubilization percentage of plant growth-promoting bacteria isolated from black pepper root. Each isolate was sequenced twice using forward and reverse primers for the 16 S rRNA gene, except for isolates Pn6, Pn11, Pn13, and Pn17. In case of conflicting results between the forward and reverse sequences, the highest identity value was taken into consideration, followed by the E-value, BLAST coverage, and subject length. Isolates were divided by family

Isolate	Taxonomic affiliation	BLAST maximum identity	BLAST coverage	Subject Accession number	ACC deaminase activity	nifH	Phosphate solubilization percentage	Accession number
Enterobacteriaceae								
Pn1	*Klebsiella pneumoniae*	100%	100%	CP054063.1	–	–	116.67%	OR842270 OR842271
Pn2	*Klebsiella quasipneumoniae*	100%	100%	CP140611.1	+	–	140.00%	OR842272 OR842273
Pn3	*Klebsiella quasipneumoniae*	99.88%	100%	MT604862.1	+	–	77.33%	OR842274 OR842275
Pn4	*Klebsiella quasipneumoniae*	100%	100%	CP140611.1	–	–	46.33%	OR842276 OR842277
Pn6	*Enterobacter* sp.	100%	100%	MK418858.1	–	–	74.67%	OR842280
Pn8	*Enterobacter cloacae*	100%	100%	MT613381.1	–	–	00.00%	OR842283 OR842284
Pn9	*Enterobacter cloacae*	100%	100%	MT613381.1	–	–	85.33%	OR842285 OR842286
Pn10	*Enterobacter bugandensis*	100%	100%	CP110983.1	–	–	155.00%	OR842287 OR842288
Pn11	*Enterobacter asburiae*	100%	100%	KU878089.1	–	–	107.33%	OR842289
Pn15	*Enterobacter cloacae*	99.86%	100%	CP056117.1	–	–	111.33%	OR842293 OR842294
Pn20	*Enterobacter roggenkampii*	100%	100%	CP133578.1	+	-	63.67%	OR842302 OR842303
Pn7	*Kosakonia sacchari*	100%	100%	MT557011.1	–	–	68.33%	OR842281 OR842282
Pn12	*Kosakonia pseudosacchari*	99.83%	100%	CP063425.1	+	+	102.00%	OR842290 OR842291
Pn16	*Kosakonia pseudosacchari*	99.87%	100%	MN607213.1	+	+	70.67%	OR842295 OR842296
Pn17	*Kosakonia sacchari*	98.80%	100%	CP040677.1	+	+	166.67%	OR842297
Pn19	*Kosakonia pseudosacchari*	100%	100%	CP063425.1	–	+	112.00%	OR842300 OR842301
Bacillaceae								
Pn18	*Bacillus cereus*	100%	100%	CP138336.1	–	-	130.00%	OR842298 OR842299
Pn21	*Bacillus paramycoides*	100%	100%	OR394251.1	–	-	57.67%	OR842304 OR842305
Unclassified								
Pn5	unclassified Bacteria	100%	100%	MK825031.1	–	–	59.33%	OR842278 OR842279
Pn13	unclassified Bacteria	100%	100%	MK825161.1	+	–	19.03%	OR842292

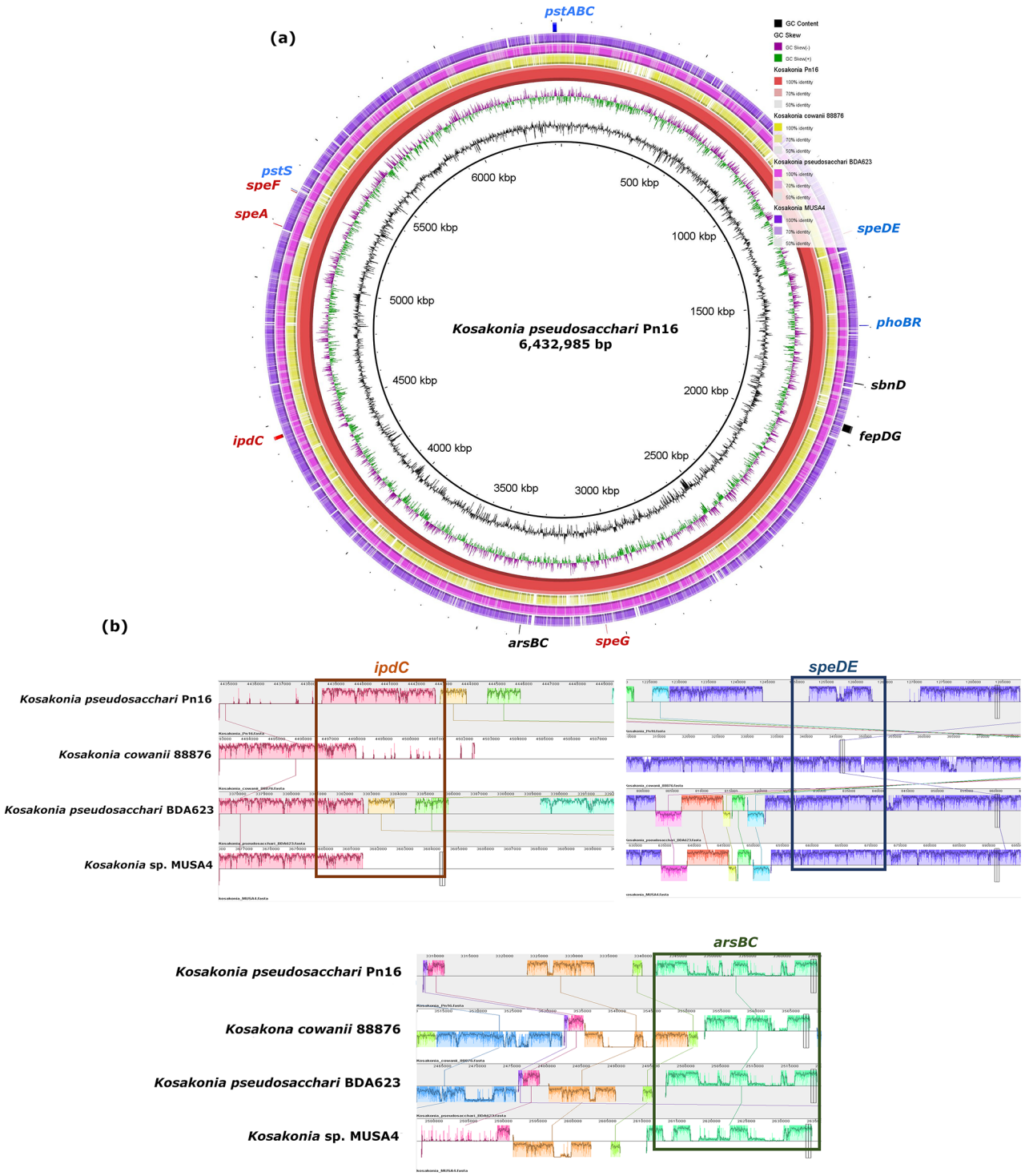

Fig. 1 Genomic ring designed in the Blast Ring Image Generator (BRIG) v.0.95. (**a**) The ring compares the genome sequences of *Kosakonia cowanii* 888 – 76 (yellow ring), *Kosakonia pseudosacchari* BDA623 (pink ring), and *Kosakonia radicincitans* MUSA4 (purple ring) against the genome of *Kosakonia pseudosacchari* Pn16 (red ring), using BLASTn. Gaps indicate regions of low similarity. (**b**) The regions of the genes *ipdC*, *arsBC*, and *speDE* are presented in detail. These regions were analyzed after mapping the contigs using CONTIGuator v.2. The top gray bar represents the *K. pseudosacchari* Pn16 genome, followed by the white and grey bars representing *K. cowanii* 888 – 76, *K. pseudosacchari* BDA623, and *K. radicincitans* MUSA4 genomes. Coding sequences (CDSs) are represented by colored graphs within the bars. Conserved CDSs are connected by colored lines. It is worth noting that *ipdC* and *speDE* genes are present only in the genome of *K. pseudosacchari* Pn16 while the arsenic resistance operon *arsBC* is conserved in all species

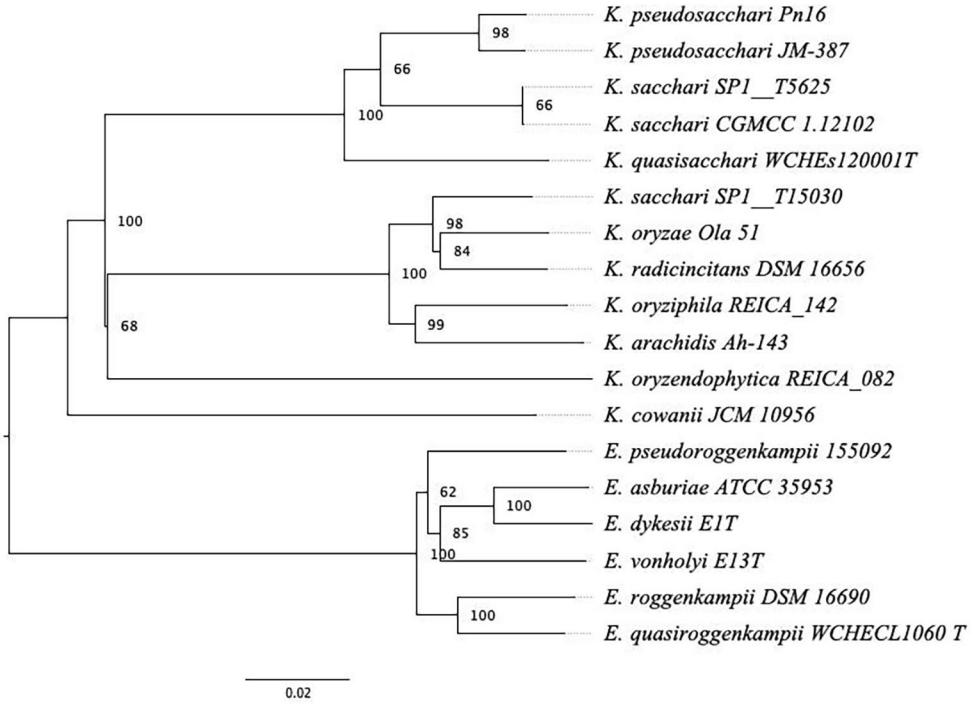

Fig. 2 Phylogenomic analysis of the *Kosakonia* genus. Analysis was performed on the Type (Strain) Genome Server (TYGS) using 11 reference genomes plus six *Enterobacter* genomes as an outgroup. *K. sacchari*, *K. pseudosacchari*, and *K. quasisacchari* grouped into a clade supported by a bootstrap value of 100, being *K. pseudosacchari* JM-387 the closest species to our isolate

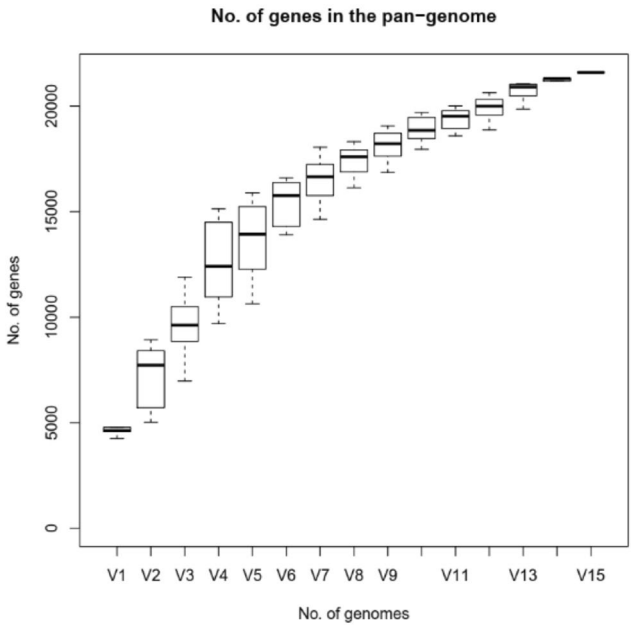

Fig. 3 Pangenome analysis performed in Roary pipeline v.3.11.2. Pan genome (21,594 gene families), core genome (789 gene families), and accessory genome (12,230 gene families) were calculated using 15 genomes of *Kosakonia* sp. deposited in GenBank. *Kosakonia pseudosacchari* Pn16 presented 523 unique genes

AntiSMASH. The phylogenomic analysis showed that *K. pseudosacchari* Pn16 clustered in a clade with the species *K. sacchari*, *K. pseudosacchari*, and *K. quasisacchari* supported by a bootstrap value of 100 (Fig. 2), being *K. pseudosacchari* JM-387 the closest species. A second clade was formed by the species *K. arachidis*, *K. oryzae*, *K. oryziphila, K. oryzendophytica*, and *K. radicincitans* (Fig. 2). *K. cowanii* was the most distant taxon within the genus. All *Kosakonia* sp. grouped together and were separated from the *Enterobacter* sp. genomes (Fig. 2). The pangenome was composed of 21,594 genes, divided into a reduced core genome of 789 genes and an accessory genome of 12,230 genes (Fig. 3). *K. pseudosacchari* Pn16 presented 523 unique genes (Table S1). Most of these genes were hypothetical proteins and transporter proteins. The Heap's Law alpha value of 0,4779 demonstrated that the genus has an open pangenome.

Several genes involved in plant-growth promotion were detected including: *speA*, *speD*, *speE*, *speG*, *speF*, *tam*, and *ipdC* (production of indole-3-acetic-acid and spermidine); *arsC*, *arsB*, *cutC*, *sbnD, corC, fepD*, and *fepG* (siderophore production and metal tolerance); *phoU, phoB, phoR, pstS, pstB, pstA*, and *pstC* (phosphate solubilization regulon); *oqxA, oqxB, mdtA*, and *mdtB* (antibiotic and biocide resistance); *nifH, nifD*, and *nifK* (nitrogen fixation). *K. pseudosacchari* Pn16 also has the entire operons for dissimilatory nitrate reduction (*narG, narH, narI*, and *narK*), nitrite reduction (*nirB, nirD, nirC*, and *CysG*), and nitric oxide reduction (*norV* and *norW*). Finally, genes involved in nitrous oxide reduction were not found.

4 Discussion

Species from the *Enterobacteriaceae* family such as *Enterobacter* sp., *Kosankonia* sp., and *Klebsiella* sp. have been described associated to roots of several plant crops such as sorghum (Silva et al. 2018) and peach palm (Silva et al. 2022). Jasim and colleagues (2013) isolated and identified twelve endophytic bacteria from *P. nigrum* L. where two of them presented 99% identity with *Enterobacter cloacae* and *Enterobacter* sp. Other PGPB isolated from black pepper include *Bacillus* sp., *Pseudomonas* sp., *Enterobacter* sp., *Pantoea* sp., *Klebisiella* sp., *Kosakonia* sp., *Micrococcus* sp., *Curtobacterium* sp., *Serratia* sp., *Acinetobacter* sp., *Brevibacillus* sp., *Proteus* sp., and *Staphylococcus* sp. (Aravind et al. 2009; Zakry et al. 2010; Toh et al. 2016; Wiratno et al. 2019; Dang and Thanh 2021). Additionally, *Klebsiella* sp., *Kosakonia* sp., and *Pantoea* sp. were also reported as potential PGPB in several other plant crops (Nascimento et al. 2015; Duarte et al. 2020). *Kosakonia* spp. were commonly found in environmental samples and have recently been recognized to interact and exert beneficial effects on plant growth (Quintas-Nunes et al. 2022).

ACC deaminase activity, nitrogen fixation, and phosphate solubilization are common characteristics of PGPB (Olenska et al. 2020) that were evaluated in our study. Several methods to analyze plant-growth promotion characteristics are used, some more analytical than others (Santoyo et al. 2019). The ACC deaminase activity and the ability to solubilize phosphate were determined using microbiological methods (Penrose and Glick 2003; Nautiyal 1999). The ability to fix nitrogen was predicted by the partial amplification of the *nifH* gene using PCR (Gaby and Buckley 2012). The ability to use ACC as the sole nitrogen source is a consequence of the ACC deaminase activity (Penrose and Glick 2003). Positive isolates for the ACC deaminase activity were affiliated to the genera *Enterobacter*, *Klebsiella*, and *Kosakonia* (Table 1). These taxa were also reported as positive for ACC deaminase activity in other studies (Nonaka et al. 2008; Jasim et al. 2013; Lau et al. 2020). Choudhury and colleagues (2021) demonstrated that enzyme activity was improved in co-cultivation with two or more PGPB. This result indicates that these isolates have potential to cleave ACC into α-ketobutyrate and ammonia, thereby reducing the amount of ACC available for ethylene biosynthesis. By doing so, ACC deaminase-producing bacteria can alleviate the negative effects of ethylene on plant growth and enhance plant tolerance to various stressors, such as drought, salinity, and heavy metals (Moon and Ali 2022).

The *nifH* gene was only detected in isolates affiliated to the *Kosakonia* sp. *K. pseudosacchari* Pn16 has two clusters of nitrogen fixation genes. They are the most efficient and widespread variant of nitrogenase, composed of a Fe protein (*nifH*) and a MoFe protein (*nifD* and *nifK*) (Gaby and Buckley 2012). The ability to fix nitrogen can enhance plant growth by providing a source of nitrogen to the plant (Santoyo et al. 2019). The primers IGK3 and DVV were identified by Gaby and Buckley (2012) as those with the best performance for the amplification of the gene in a wide range of taxa. The *nifH* gene has been widely used in culture-independent studies on nitrogen-fixing bacteria (Liao and Inglett 2014; Moseman-Valtierra et al. 2016) and is considered one of the best genetic markers to identify diazotrophs (Meng et al. 2019). The ability to fix nitrogen was previously reported in free-living *Kosakonia* sp. (Chen et al. 2020; Bar-Shmuel et al. 2020). Meng and colleagues (2015) identified six isolates of root associated *Kosakonia* that were positive for the *nifH* gene. It is worth noting that PCR method may fail to detect *nifH*, mainly due to the presence of phylogenetically distant nitrogen-fixing enzymes (Islam et al. 2007). *K. pseudosacchari* Pn16 also presented genes involved in other processes of the nitrogen cycling (Fig. 4). Dissimilatory nitrate reduction, catalyzed by the periplasmic enzyme Nap or the membrane-bound cytosolic enzyme Nar, is a crucial step that controls the bioavailability of nitrate in several ecosystems (Asamoto et al. 2021). *K. pseudosacchari* Pn16 carries Nar-mediated dissimilatory-nitrate reduction genes (Fig. 4), which emphasizes its importance not only for biological fixation of N but in maintaining the bioavailability levels of nitrate in the soil. Nitrate can be reduced to ammonium or denitrified to N_2. *K. pseudosacchari* Pn16 does not have the genes for dissimilatory nitrate reduction to ammonium. However, it has an almost complete denitrification pathway starting at the nitrite reduction (*nir* operon) followed by nitric oxide reduction (*nor* operon) but does not have genes for reduction of nitrous oxide to N_2 (Fig. 4).

Compared to other *Kosakonia* species, *K. pseudosacchari* Pn16 presented key genes for promotion of plant growth. For example, *K. pseudosacchari* Pn16 and *K. radicincitans* DSM 16,656 shared genes related to phosphate solubilization such as the *pho* and *pst* genes, as well as enzymes with ACC deaminase activity, such as the *spe* gene. Regarding the production of siderophores, the only set of genes shared between the species was the Ferric Enterobactin Transporter (Fep) (Berger et al. 2017). Additionally, *K. pseudosacchari* Pn16 carries genes for flagellum formation and three types of secretion systems: type I (TISS), type II (TIISS), and type III (TIIISS). TIIISS is crucial for the establishment of symbiosis in bacteria of the genus *Rhizobium* and for colonization of the plant rhizosphere by saprophytic *Pseudomonas* (Viprey et al. 2002; Rainey et al. 2002).

The best phosphate solubilizing species were the same found by Silva and colleagues (2018) in sorghum including *Klebsiella* sp., *Pantoea* sp., *Enterobacter* sp., and *Kosakonia*

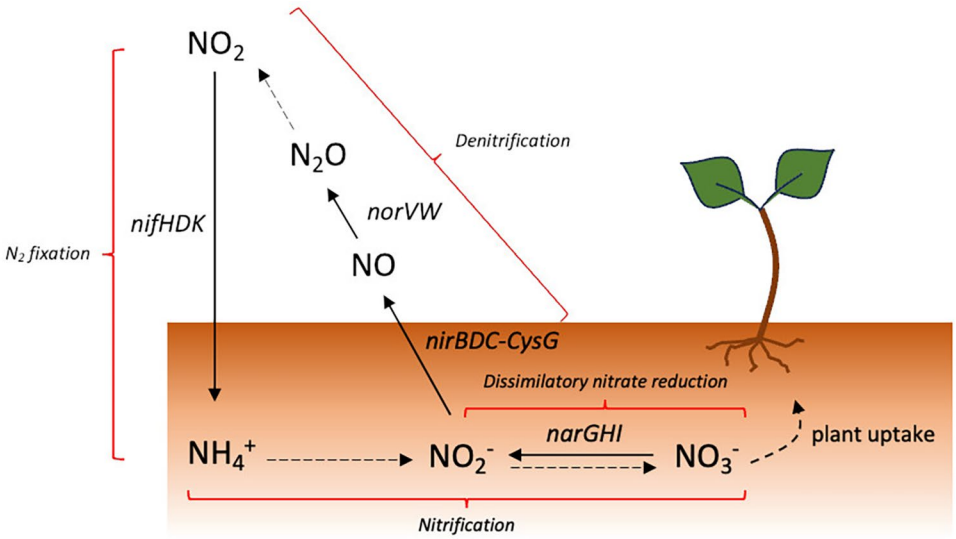

Fig. 4 Schematic representation of the nitrogen cycle pathways found in the ***K. pseudosaccharii*Pn16 genome.** Genes that encode enzymes are shown next to the arrows that connect the reaction's substrate and product. Dotted arrows represent reactions catalyzed by enzymes that were not found in the bacterial genome. *K. pseudosacchari* Pn16 has two nitrogenase clusters (*nifHDK*). In addition, the bacterium contains genes involved in the dissimilatory nitrate reduction pathway and an almost complete denitrification pathway

sp. Dang and Thanh (2021) found isolates from black pepper affiliated to the genus *Bacillus* with high capacity of solubilization, ranging from 2.75 up to 61.88 mg of phosphorus pentoxide by 1 L of medium after five days of incubation. Recent works have highlighted the plant-growth promotion potential of the genus *Kosakonia.* Several members of this genus are endophytes of different agricultural plants and demonstrated important features such as IAA production, phosphate solubilization, and antimicrobial activity against plant pathogens such as *Botrytis* spp. and *Phytophthora* spp. (Olanrewaju et al. 2017; Romano et al. 2020). However, the genus *Kosakonia* still remains largely unexplored, especially its genetic characteristics (Romano et al. 2020).

The phylogenetically closest species to our isolate was *K. pseudosacchari* JM-387, a beige pigmented strain, isolated from field-grown corn root tissue in Tallassee, Alabama (Kämpfer et al. 2016). The ANI analysis showed 98.75% identity between *K. pseudosacchari* Pn16 and other *K. pseudosacchari* genomes. Our isolate has a genome larger than the other four *K. pseudosacchari* deposited in GenBank (GCA_015167415.1, GCA_027912575.1, GCA_900184035.1, GCA_002510255.1). Ten Symbiotic Islands were detected by GIPSy with an average size of 12,445 bp. Several important genes were detected in these islands such as the formate dehydrogenase (*fhdS*) enzyme gene that catalyzes the oxidation of formate to carbon dioxide coupled to the reduction of NAD^+ to NADH (Hatrongjit and Packdibamrung 2010). This enzyme plays an important role in cell energy supply and was found in several organisms from bacteria to plants (David et al. 2010). Genes related to the production of siderophores such as *sbnD*, *fepD*, and *fepG* were also detected. Production of siderophores is a very important characteristic for PGPB (Chouhan et al. 2021). They are organic molecules with low molecular weight able to chelate and solubilize Fe ions and other metals, making them available for transport through biological membranes (Rajkumar et al. 2010). Iron has a low bioavailability in the environment. For example, in basic or neutral pH, Fe is found in its insoluble and oxidized form of Fe^{3+} (Kramer et al. 2020). Therefore, the biological solubilization of Fe through siderophores is an indirect mechanism for plant growth promotion. Additionally, proteins from the Resistance-Nodulation-Division (RND) family transporters such as *mdtB* and *mdtC* were also found in the Symbiotic Islands. These transporter proteins pump out a wide range of inhibitors including antibiotics and biocides (Kim et al. 2010), contributing to the bacterial growth and survival in the soil.

AntiSMASH predicted 28 regions of potential BGCs (Table S2). Nineteen of these 28 BGCs were related to saccharide production. Among these, we highlight the exopolysaccharides (EPS) which are commonly involved in biofilm formation. Additionally, EPS forms aggregates with soil particles, binds to ions and consequently reduces soil salinity, resulting in an increased bioavailability of water and nutrients in the rhizosphere (Nunkaew et al. 2015). This is an extremely interesting characteristic for combating water stress (Upadhyay et al. 2011). The BGC 20 showed 91% identity with colonic acid-producing genes, a loosely associated EPS mesh that is commonly found in enterobacteria. Genes for surfactin production were detected in region 17. Surfactin is a cyclic lipopeptide that acts as a biosurfactant which has demonstrated inhibitory activity against several plant pathogens (Bais et al. 2004). The amphiphilic molecule binds to the cell membrane causing disruption and cell death (Blake et al. 2021). This finding demonstrates the potential use of *K. pseudosacchari* Pn16 as a possible biocontrol agent. The other clusters were related to fatty acid production (4 BGCs), nonribosomal peptide synthetase (NRPS) (2 BGCs), ribosomally synthesized and post-translationally

modified peptides (RiPPs) (1 BGC), arylpolyene (1 BGC), and thiopeptide (1 BGC) (Table S2). Fifteen of the 28 BGCs presented 0% similarity with known clusters. The strains BDA62-3 and RX.G5M8 have complete genomes deposited in GenBank. Both have 20 BGCs predicted by antiSMASH. So far, *K. pseudosacchari* Pn16 is the strain of the species with the highest number of BGCs.

Additionally, an important operon of the Pho regulon was found, composed by the genes *pstSCAB-phoU* and the regulatory genes *phoRB*. These genes are involved in the process of solubilization and transport of organic or inorganic phosphate (Timofeeva et al. 2022). The Pho regulon is mainly activated at low concentrations of phosphate (Santos-Beneit et al. 2015). It has a crucial role in promote plant growth since it is capable of enhance phosphate availability to the plant. This *in silico* prediction corroborates the results of the phosphate solubilization assay. We also found genes related to the production of indole acetic acid (IAA), spermidine and polyamine. IAA is an important plant hormone that regulates several aspects of plant growth and development, including resistance to environmental stressors (Bianco et al. 2009). IAA is produced by microorganisms through tryptophan-dependent or -independent pathways (Tang et al. 2023). *K. pseudosacchari* Pn16 has two genes of the indole-3-pyruvic acid (IPA) pathway: *tam* and *ipdC*. This is one of the major pathways for microbial IAA biosynthesis (Tang et al. 2023). *Bacillus thuringiensis* RZ2MS9 mutants lacking the ability to produce IAA have significantly reduced ability to promote maize growth compared to the wild-type strain (Figueredo et al. 2023). Additionally, IAA-producing bacteria can promote plant growth indirectly by improving plant-resistance to abiotic stresses. For example, soybean shows a significantly improvement in growth under salt stress when inoculated with the indole acetic acid-producing *Acinetobacter pittii* YNA40 (Kang et al. 2023).

The *oqxAB* multidrug efflux pump genes were detected by ResFinder. This efflux pump usually confers resistance to multiple drugs such as trimethoprim, ciprofloxacin, nalidixic acid, benzalkonium chloride, chloramphenicol, and cetylpyridinium chloride (Kim et al. 2010). This system is found either on the chromosome or on large plasmids (Li et al. 2019). Therefore, several characteristics present in bacterial species known to promote plant growth such as *K. radicincitans* (Berger et al. 2017)d *velezensis* (Zaid et al. 2022) were found in *K. pseudosacchari* Pn16.

Since the genomic information for the genus is relatively scarce, we calculated the pangenome using 15 genomes of *Kosakonia* available in the GenBank database. The results indicated an open pan genome with a small number of gene families in the core genome (Fig. 3). An expected result given the low number of available genomes and the genetic diversity of the taxon. Ninety-six (18%) of the unique genes were annotated as hypothetical proteins. This exemplifies the lack of knowledge about the genomic content of the species. Regarding the genes involved in plant-growth promotion, *speD, phoR, phoB, narG*, and *norR* were among the unique genes of *K. pseudosacchari* Pn16 (Table S1), which reinforces its potential for promoting plant growth. By comparing unique genes to the KEGG dataset using blastKOALA, the top three functions found were: genetic information processing, carbohydrate metabolism, and signaling and cellular processes. Another 15 functions were also detected in this dataset (Figure S1).

5 Conclusions

In this study, 20 endophytic bacterial strains were isolated from black pepper roots and subsequently evaluated for 1-aminocyclopropane-1-carboxylate deaminase activity, nitrogen fixation, and phosphate solubilization. Three isolates affiliated to the *Kosakonia* genus exhibited positive results in all assays. The genomic analysis of *K. pseudosacchari* Pn16 revealed key genes related to the production of indole acetic acid, spermidine, exopolysaccharides, biosurfactants, and siderophores. According to the genetic content, *K. pseudosacchari* Pn16 can fix nitrogen and plays an important role in maintaining nitrate bioavailability in the soil. These findings highlight the potential of *K. pseudosacchari* Pn16 for the development of biofertilizers, offering a sustainable alternative to chemical fertilizers and mitigating environmental risks.

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Author Contributions AMS, JKRS, and RAB conducted the conceptualization, data curation, methodology, and validation. AMS, JKRS, RAB, SSC, and DAG wrote the original draft of the manuscript; AMS, PCPN, SSC, DAG, JKRS, and RAB conducted all the investigation and formal analysis under supervision the RAB, DAG, and JKRS; SSC conducted the software, and visualization; AS, MPCS, and RAB conducted the conceptualization, funding acquisition, and project administration. All authors read and approved the final manuscript.

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Data Availability Genome sequence is available on GenBank under accession number PRJNA900184. 16 S rRNA gene sequences are available on GenBank under the accession numbers provided in Table 1.

Declarations

Competing Interests All authors declare that they have no conflicts of interest.

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