Original article

Bioremediation potential of glyphosate-degrading microorganisms in eutrophicated Ecuadorian water bodies

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ABSTRACT

Phosphonate compounds are the basis of many xenobiotic pollutants, such as Glyphosate (N-(phosphonomethyl-glycine). Only procaryotic microorganisms and the lower eukaryotes are capable of phosphonate biodegradation through C–P lyase pathways. Thus, the aim of this study was to determine the presence of C–P lyase genes in Ecuadorian freshwater systems as a first step towards assessing the presence of putative glyphosate degraders. To that end, two Nested PCR assays were designed to target the gene that codifies for the subunit J (phnJ), which breaks the C-P bond that is critical for glyphosate mineralization. The assays designed in this study led to the detection of phnJ genes in 7 out of 8 tested water bodies. The amplified fragments presented 85–100% sequence similarity with phnJ genes that belong to glyphosate-degrading microorganisms. Nine sequences were not reported previously in the GenBank. The presence of phosphonate degraders was confirmed by isolating three strains able to grow using glyphosate as a unique carbon source. According to the 16S sequence, these strains belong to the Pantoea, Pseudomonas, and Klebsiella genera. Performing a Nested PCR amplification of phnJ genes isolated from eutrophicated water bodies, prior to isolation, may be a cost-effective strategy for the bioprospection of new species and/or genes that might have new properties for biotech industries, laying the groundwork for additional research.

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1. Introduction

Phosphonates are a class of organophosphorus compounds with a C-P bond that are widely used in different fields of human economic activity (Rott et al., 2018). These compounds are the basis of many xenobiotic pollutants, for instance glyphosate. This herbicide controls weeds by inhibiting the action of the enzyme 3-enolpyruvylshikimic acid 5-phosphate synthase (EPSPS), which is necessary for the synthesis of aromatic amino acids in plants and microorganisms (Rueppel et al., 1977).

Nevertheless, glyphosate has been reported to cause harmful effects on mammals, birds, amphibians, and fish (Matozzo et al., 2020; Janßen et al., 2019). Several reports have highlighted its

potential risk to human health, such as cardiotoxicity, carcinogenicity (Guyton et al., 2015), endocrine disruption (Mesnage & Antoniou, 2017), skin toxicity (Amerio et al., 2004; Hindson & Diffey, 1984), and reproductive disorders (Avila-Vazquez et al., 2018). It may remain suspended in dust particles for up to 72 h after being applied as a spray (Ganzelmeier et al., 1995). After that, around 52% of glyphosate reaches surface water bodies (Tsui & Chu, 2004). Once it reaches aqueous media, its half-life can vary from 63 days (shallow waters) to a maximum of 70 days (pond water) (Szekacs & Darvas, 2012). It has also been established that glyphosate is accumulated for periods longer than 4 years when leached into groundwater (Okada et al., 2019). Glyphosate stimulates eutrophication by increasing total phosphorus, favoring the bloom and toxin production of cyanobacteria, thereby affecting water quality (Bastos Gonçalves et al., 2020).

Only procaryotic microorganisms and the lower eukaryotes are capable of phosphonate biodegradation via several pathways. Heterotrophic aerobic/facultative microorganisms capable of biodegrading glyphosate, such as Pseudomonas spp., Arthrobacter spp., Enterobacter aerogenes, Geobacillus caldoxylosilyticus, Flavobacterium spp., and Bacillus spp., have been identified in soil/sediment (from water) and sludge-activated technology (Balthazor & Hallas,

1986; Ermakova et al., 2010; Solomon & Thompson, 2003; Wijekoon & Yapa, 2018). Through their metabolic actions, they are major players in terms of environmental sustainability. Destruction of the C–P bond by the C–P lyase pathway is of fundamental importance, and understanding the process is a basic problem within the study of the biochemistry and physiology of microorganisms. This pathway is encoded by *phnCDEFGHIJKLMNOP* operon, where the *phnJ* subunit is the key enzyme of the complex. It catalyzes the cleavage of the C-P bond and is also highly similar at the amino acid level to various microorganisms (Stosiek & Klimek-ochab, 2019). The detection of *phnJ* genes and new alleles by molecular approaches might provide a source of potential genetic resources that could lead to the isolation of many novel biocatalysts and a variety of other molecules with a high potential for downstream applications.

Given the difficulty of bringing most bacteria into pure culture, a survey of the presence of sequence variants might be a viable first step prior to isolation. The samples should be taken from an environment polluted with phosphonates, since the effect of exposure to contaminants on the structure of microbial communities favors degrading microorganisms (Maier & Gentry, 2015). Adaptation of microbial populations most commonly occurs by the induction of enzymes necessary for biodegradation, followed by an increase in the population of biodegrading organisms (Leahy & Colwell, 1990). One strategy for detecting the presence of these microorganisms would be to amplify gene targets directly from environmental DNA from polluted sites using generic primers, followed by cloning and sequencing (Roh et al., 2006; Gillings et al., 2008; Hundermark & Takahashi, 2018). The detection of *phnJ* may be achieved by PCR using environmental DNA (eDNA) extracted from water samples as a template (Seymour, 2019). Nevertheless, the study of functional genes in microbial communities from eDNA could be a challenging task, since the concentration of target DNA could be on undetectable ranges for conventional molecular methods, such as end-point PCR. In the literature, primers designed for the amplification of the *phnJ* subunit of the C-P lyase multienzymatic complex are focused on the quantitative polymerase chain reaction (qPCR) technique (Morales et al., 2020; Firdous et al., 2020; Wang et al., 2017; Yao et al., 2016; Mauffrey et al., 2017). Among these authors, Morales et al. (2020) designed primers to amplify environmental samples to quantify the *phnJ* gene, although not from water but soil. Mauffrey et al. (2017) used primers designed by Parker et al. (1999) that were tested in water environmental samples but just for phnGIHK genes from *Rhizobium meliloti*. Other authors used primers to quantify *phnJ* in bacterial cultures (Firdous et al., 2020; Wang et al., 2017; Yao et al., 2016). Thus, previous works do not provide methods to broadly assess the presence of *phnJ* from a wide range of microorganisms in water environmental samples.

Since glyphosate is widely used in Ecuador (Paz-y-miño et al., 2007; Benner et al., 2016; Hurtig et al., 2003), we hypothesized that there would be microorganisms capable of degrading this herbicide in water bodies affected by agricultural lixiviate. Consequently, this research aimed to implement a methodology based on Nested PCR to identify the presence of potential glyphosate-degrader microorganisms in water bodies from the Andean and Amazonian regions of Ecuador as an initial step prior to strain isolation

2. Materials and methods

2.1. Study area

Eight study areas were chosen for this research (Table 1). (i) Yahuarcocha is located in Imbabura province. It is a eutrophic

lagoon since it is a recipient of wastewater discharges, fertilizers, and cattle grazing waste (Echeverría-Almeida & Athens, 2016). (ii) San Pablo is a weak-moderated eutrophic lake also located in Imbabura. Its poor condition is due to the presence of chemical residues used in plant fertilization and livestock care (Gómez, 2017). (iii) Limoncocha Lagoon is in the Limoncocha Biological Reserve in the southwestern region of Sucumbios province. This eutrophic water body receives domestic wastewater and agricultural leachate (Carrillo et al., 2021). (iv) Machángara River originates in the Atacazo Highlands, located 6.5 km from the southern outskirts of Quito. It crosses the city, eventually reaching the Tumbaco valley. Moreover, it receives most of the cityś wastewater (Voloshenko-Rossin et al., 2015). (v) Cuyabeno is a lagoon that straddles Sucumbios and Orellana provinces and is surrounded by rainforest (Mestanza et al., 2019). (vi) Numbayme River is situated in Morona Santiago province. This water body presents a high amount of organic and inorganic matter due to its proximity to slaughterhouses that throw pig feces into its waters as well as waste of various kinds, such as plastics, bottles, and rags (Quishpi, 2014). vii) Pita River is part of the upper basin of the Guayllabamba River and one of the main water sources that supply drinking water to the Metropolitan District of Quito. It is in Pichincha province and is increasingly influenced by anthropogenic activities like water collection for irrigation or purification, direct discharges of sewage, and waste from urban and industrial drainage basins, livestock, and agricultural and floricultural activities, among others (Pérez, 2007). viii) Yambo is a lagoon located in Cotopaxi province, and is considered eutrophic since it is a direct recipient of waste from nearby private complexes and the devastation of nearby vegetation (Orquera & Cabrera, 2020).

2.2. Sample collection and DNA isolation

Two liters of water were collected in sterile plastic bottles, kept in an iced cooler, and taken to the laboratory for further analysis.

Water samples were filtered with 0.45 μm nitrocellulose membrane filters for further analysis. Residues attached to membrane filters were submitted to DNA extraction with PureLinkTM Microbiome DNA Purification Kit (Thermo Fisher), following the manufactureŕs instructions. DNA quantification and quality were checked using a Nanodrop™ 2000. Aliquots were stored at −20 °C until analysis.

2.3. Nested PCR primer design

A total of 43 non-redundant sequences of *phnJ* genes were downloaded in FASTA format from the GenBank database at the NCBI (National Center for Biotechnology information). These included sequences belonging to the two following classes (with family names in parentheses): Gammaproteobacteria (Enterobacteriaceae, Vibrionaceae, and Pseudomonadaceae) and Alphaproteobacteria (Rhizobiaceae, Bradyrhizobiaceae, Phyllobacteriaceae, Rhodobacteraceae, and Nitrobacteraceae). The sequences were aligned with MEGA X software (Kumar et al., 2018) using the MUSCLE algorithm to identify the consensus sequence. Conserved regions were identified by means of visual inspection. Due to the variability found among the sequences, two different sets of primers were designed and checked using the IDT oligo analyzer, which was also used to verify the behavior and properties of the designed primers. Set 1 was designed close to the 5́end of the *phnJ* gene and Set 2 was located close to the 3́end. The two amplicons overlap in a region of 200 bp. In order to perform a Nested PCR on each set, we included three primers. Set 1 comprised the outer primers Fphnj301 (forward)/Rphnj302 (reverse) and the inner primer Rphnj 301(reverse), and Set 2 the outer Fphnj201 (forward)/ Rphnj201 (reverse) and the inner Fphnj (forward) (Table 2;

Table 1
Geographic location of study sites.

Water body	Province	Latitude	Longitude	Altitude (m.a.s.l)
Yahuarcocha Lagoon	Imbabura	0°22′34.3164″N	78°6′32.3424″W	2191
San Pablo Lake	Imbabura	0°12′21″N	78°13′09′W	2700
Limoncocha Lagoon	Sucumbíos	0°24′17.5″S	76°37′05.9 W	229
Machángara River	Pichincha	0°15′ 22.97″ S	78°31′ 31.9″ W	2850
Cuyabeno Lagoon	Sucumbíos	0°07′00″S	75°50′00″W	280
Numbayme River	Morona Santiago	1°42′38.664″S	78°0′ 3.3192″W	886.4
Pita River	Pichincha	0°36′30.6″S	78°24′14.4″W	2536
Yambo Lagoon	Cotopaxi	1°06′04.9″S	78°35′20.00″W	2582

Table 2
Properties of primers designed for Nested PCR assays. Bold letters indicate the degenerated bases in each primer; Tm = melting temperature; (i) = inner primer designed for second PCR.

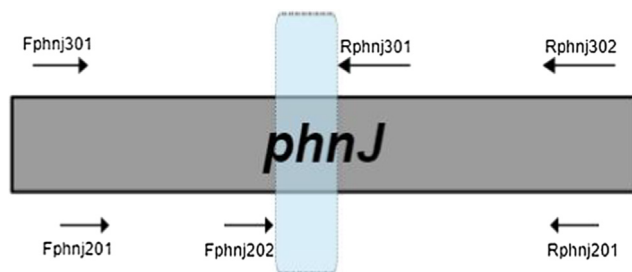| Code	Primers (5′→ 3′)	Tm (°C)	G/C (%)	Position
Set 1	Fphnj301 TACAACTTCGCCTATCTGGACG	56.4	50	19–97
	Rphnj301(i) TT**S**GG**R**TTGTCGAATTTCGG	54.4	47.5	584–662
	Rphnj302 GTGTCGGAGCAGACGAACAT	57.4	55	806–884
Set 2	Fphnj201 GGCTA**Y**AACTTTGGCTATCT**S**G	55.7	47.7	37–46
	Fphnj202(i) AGACCCG**Y**CACCGCATCC	60.2	69.4	342–386
	Rphnj201 CAATA**R**TCGGT**R**TCGGAGCAGAC	59.4	47.8	836–882

Fig. 1. Scheme of the gene and primer annealing locations. The rectangle in the middle represents the overlapped area between PCR products from Set 1 and Set 2.

Fig. 1). External primers were designed to amplify 700–1000 bp regions and internal primers to amplify 500–600 bp regions.

2.4. Analysis of environmental DNA

To assess the absence of inhibition in the PCR reaction, the amplification of the region 16S rDNA gene was carried out as a positive control using the primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 519R (5′- GTNTTACNGCGGCKGCTG-3′) (Heuer et al., 1997).

Primers for *phnJ* gene detection were tested on environmental genomic DNA from the previously mentioned study areas. Two consecutive PCRs for each primer set were performed. Amplification was carried out in reactions of 25 μL containing 2X GoTaq® Green Master Mix (Promega Corporation), 0.25 μM of each primer, and 25–35 ng of extracted eDNA. The first PCR was performed for 30 cycles as follows: initial denaturation step at 95 °C for 2 min, subsequent denaturation step at 95 °C for 0:45 min, annealing step at 56.4 °C (Fphnj301/Rphnj302) and 55.8 °C (Fphnj201/Rphnj201) for 0:45 min, extension step at 72 °C for 1 min, and final extension for 2 min at 72 °C. One microliter of the amplified product was used as a DNA template for the second PCR.

The second round of amplification in the Nested PCR protocol was performed for 25 cycles as follows: initial denaturation step at 95 °C for 2 min, subsequent denaturation step at 95 °C for 0:45 min, annealing step at 56.4 °C (Fphnj301/Rphnj301(i)) and 60 °C (Fphnj202(i)/Rphnj201) for 0:45 min, extension step at 72 °C for 0:30 min, and final extension for 2 min at 72 °C.

The visualization of the amplified products was carried out by electrophoresis with agarose gels at 1.5% stained with 1/10000 v/ v of SYBR™ Safe DNA Gel Stain (ThermoFisher) under UV light using the automatic option of BioRad ChemiDoc™ MP Imaging System photo documenter. The amplicons were purified using AccuPrep PCR/Gel Purification Kit (Bioneer) and sequenced at the Sequencing service of Universidad de Las Américas (Quito, Ecuador).

2.5. Cloning

In those cases where the direct sequencing of the PCR product resulted in an ambiguous chromatogram with overlapping peaks, cloning of the PCR product was performed. The PCR products were introduced to a pCR™4-TOPO® TA vector, following TOPO™ TA Cloning™ Kit for Sequencing protocol. Ligation products (TOPO® TA with inserts) were used to transform chemically competent *E. coli* DH5α cells, as mentioned by Hanahan (1983). Positive colonies were checked by PCR with M13 forward and reverse primers provided by the kit, and plasmids were then extracted by alkaline lysis (Bimboim & Doly, 1979).

2.6. Sequence analysis

Sequences were edited in MEGA X software (Kumar et al., 2018), and compared against the GenBank database at the NCBI using Basic Local Alignment Tool (BLASTn and BLASTx) (McGinnis & Madden, 2004).

The sequences obtained from eDNA were registered in the NCBI nucleotide database under the following accession numbers: MZ423873- MZ423888.

A phylogenetic tree was built with the *phnJ* sequences obtained and *phnJ* sequences retrieved from Genbank databases. The sequences were aligned with MEGA X software (Kumar et al., 2018) using the MUSCLE algorithm. A best-fit model was chosen based on the Akaike Information Criterion (AIC) using the option for the best DNA/protein model implemented in MEGA X. Tree topologies and branch lengths were computed with the maximum-likelihood method (ML), with 500 bootstrap replicates, using General Time Reversible with a gamma distribution.

2.7. Isolation of microbial glyphosate-degrading bacteria

Glyphosate-degrading bacteria were isolated in mineral salt medium 1 (MSM1) (Benslama & Boulahrouf, 2013). About 10 mL of Machángara River water sample was added into 90 mL MSM1

in 250 mL Erlenmeyer flask with the addition of 1 g/L of glyphosate and incubated for 72 h in the dark at 30 °C ± 2 under shaking conditions (150 rpm). A 5 mL volume of the previous medium was transferred into 100 mL fresh MSM1 containing 3 g/L of glyphosate and incubated for 72 h then transferred into fresh MSM1 medium containing 6 g/L of glyphosate for 72 h (Manogaran et al., 2017). Serial culture dilutions were plated on solid MSM1 supplemented with 1 g/L of glyphosate and incubated at 30 °C for 72 h. The pure strains obtained were streaked on Nutrient Agar for further analysis.

Furthermore, isolated microorganisms were streaked by triplicate on MSM2 for a sole phosphorus source assay (Manogaran et al., 2017), and Czapek Dox medium (CZN) for a sole nitrogen source assay (Carranza et al., 2017). Incubation conditions were 30 °C for 48–72 h. Media without glyphosate were used as a negative control.

phnJ gene from isolates was amplified and sequenced. The sequences obtained were registered in the NCBI nucleotide database under the numbers OK648466-8.

3. Results

Universal primers for *phnJ* gene amplification by Nested PCR were designed. To achieve this goal, a total of 43 *phnJ* sequences from 29 genera were aligned to find conserved regions. The mean variability (p-distance) among the retrieved sequences was high (0.263). This fact made the selection of universal functional primers difficult. Therefore, two sets of primers were designed for Nested PCRs. Set 1 comprised Fphnj301 (forward), Rphnj302 (re-

verse), and the internal Rphnj 301 (reverse), while Set 2 consisted of Fphnj201 (forward), Rphnj201 (reverse), and the internal Fphnj202 (forward) (Table 2; Fig. 1). Both Nested PCRs amplified a 550–600 bp fragment. The resultant amplicons overlapped a region of 200 bp that allowed us to be sure that we had amplified the same haplotype with a different set of primers.

The eDNA from eight different water bodies was used as a template for PCR to assess the occurrence of *phnJ* genes for glyphosate degradation. The concentration of DNA obtained from water environmental samples was variable, ranging from 5 to 100 ng/μL. All the samples were positive for 16S amplification. In general, the PCR performed with the external primers did not amplify a detectable band (Figure S1). Nevertheless, Nested PCR assays with both sets of primers yielded positive amplicons of the expected size (Figure S1). Assays using Set 1 primers yielded amplicons for all eDNAs except those from Numbayme River and Yambo Lagoon, and Set 2 assays did not yield amplicons for San Pablo Lake, Cuyabeno, and Numbayme water samples (Table 3).

PCR amplifications were submitted to sequencing in order to confirm effective *phnJ* gene detection. The direct sequencing of the amplicons was successful in most cases, rendering a clear chromatogram, hence the presence of a single amplicon was assumed. However, direct sequencing of the amplicons from Machángara, Cuyabeno, and Yambo samples led to overlapping peaks in the sequence data, so the Nested PCR amplification was cloned before sequencing. A total of 16 different sequences were obtained, eight of them with Set 1 (named *phnJ1.1–1.8*) and the other eight with Set 2 (*phnJ2.9–2.16*). The source of each sequence is shown in Table 2. The overall p-distance was 0.22 among Set 1 sequences and 0.25 among Set 2 sequences.

Table 3
PCR amplification results for each set of primers and sequences obtained for each water sample.

Water body	Set 1			Set 2		
	PCR	Sequences	Accession number	PCR	Sequences	Accession number
Cuyabeno Lagoon	+	*phnJ1.4 phnJ1.5*	MZ423876 MZ423877	–	–	–
Limoncocha Lagoon	+	*phnJ1.2*	MZ423874	+	*phnJ2.12*	MZ423884
Machángara River	+	*phnJ1.3 phnJ1.5 phnJ1.6*	MZ423875 MZ423877 MZ423878	+	*phnJ2.9*	MZ423881
Numbayme River	–	–		–	–	
Pita River	+	*phnJ1.8*	MZ423880	+	*phnJ2.10*	MZ423882
San Pablo Lake	+	*phnJ1.1*	MZ423873	–	–	
Yahuarcocha Lagoon	+	*phnJ1.7*	MZ423879	+	*phnJ2.13*	MZ423885
Yambo Lagoon	–	–	–	+	*phnJ2.11 phnJ2.14–16*	MZ423883 MZ423886-8

Table 4
BLASTx analysis of sequences detected from different study areas.

Sequence	Identity (%)	Closest match (protein)	Accession number	E- Value
phnJ1.1	99.32	alpha-D-ribose 1-methylphosphonate 5-phosphate C-P-lyase PhnJ [unclassified *Agrobacterium*]	WP_069042816.1	6e-96
phnJ1.2	94.59	alpha-D-ribose 1-methylphosphonate 5-phosphate C-P-lyase PhnJ [*Pseudorhodobacter* sp. MZDSW-24AT]	WP_100197611.1	4e-82
phnJ1.3	100	alpha-D-ribose 1-methylphosphonate 5-phosphate C-P-lyase PhnJ [*Ensifer*]	WP_034800089.1	1e-96
phnJ1.4	87.16	carbon-phosphorus lyase [*Rhodospirillaceae bacterium*]	HBC08678.1	7e-82
phnJ1.5	85.71	alpha-D-ribose 1-methylphosphonate 5-phosphate C-P-lyase PhnJ [*Azospirillum* sp. TSO22-1]	WP_109119961.1	4e-83
phnJ1.6	84.46	alpha-D-ribose 1-methylphosphonate 5-phosphate C-P-lyase PhnJ [*Azospirillum* sp. TSO22-1]	WP_109119961.1	6e-82
phnJ1.7	92.57	carbon-phosphorus lyase [*Rhodopseudomonas* sp.]	MBI1204102.1	5e-89
phnJ1.8	95.95	alpha-D-ribose 1-methylphosphonate 5-phosphate C-P-lyase PhnJ [*Tabrizicola piscis*]	WP_125326459.1	2e-82
phnJ2.9	98.53	alpha-D-ribose 1-methylphosphonate 5-phosphate C-P-lyase PhnJ [*Pseudomonas toyotomiensis*]	WP_059391588.1	4e-77
phnJ2.10	100	alpha-D-ribose 1-methylphosphonate 5-phosphate C-P-lyase PhnJ, partial [*Escherichia coli*]	EFD7715694.1	4e-78
phnJ2.11	99.26	alpha-D-ribose 1-methylphosphonate 5-phosphate C-P-lyase PhnJ [*Roseococcus thiosulfatophilus*]	WP_206930585.1	2e-76
phnJ2.12	97.06	alpha-D-ribose 1-methylphosphonate 5-phosphate C-P-lyase PhnJ [*Rhodobacteraceae bacterium*]	MBD1203802.1	1e-73
phnJ2.13	100	alpha-D-ribose 1-methylphosphonate 5-phosphate C-P-lyase PhnJ, partial [*Escherichia coli*]	EFD7715694.1	4e-78
phnJ2.14	91.18	alpha-D-ribose 1-methylphosphonate 5-phosphate C-P-lyase PhnJ [*Roseomonas eburnea*]	WP_211847275.1	7e-71
phnJ2.15	89.71	alpha-D-ribose 1-methylphosphonate 5-phosphate C-P-lyase PhnJ [*Rhodospirillales bacterium*]	MBN8892659.1	3e-70
phnJ2.16	91.18	alpha-D-ribose 1-methylphosphonate 5-phosphate C-P-lyase PhnJ [*Rhodospirillales bacterium*]	MBN8892659.1	2e-72

All the sequences showed similarity with published C-P lyase protein sequences in GenBank, seven of them had a high identity (over 97%) with species well characterized as glyphosate biodegraders (Table 4). The *phnJ2.10* and *phnJ 2.13* sequences had only one nucleotide mismatch leading to the same amino acid sequence, 100% identical to the *phnJ* gene of *Escherichia coli*.

Two phylograms were constructed in order to analyze the relationships between the *phnJ* sequences obtained from the Ecuadorian water bodies and their closest matches retrieved in the Blast analysis.

According to the phylogram constructed with sequences amplified with Set 1 primers, the sequences clustered with genes of spe-
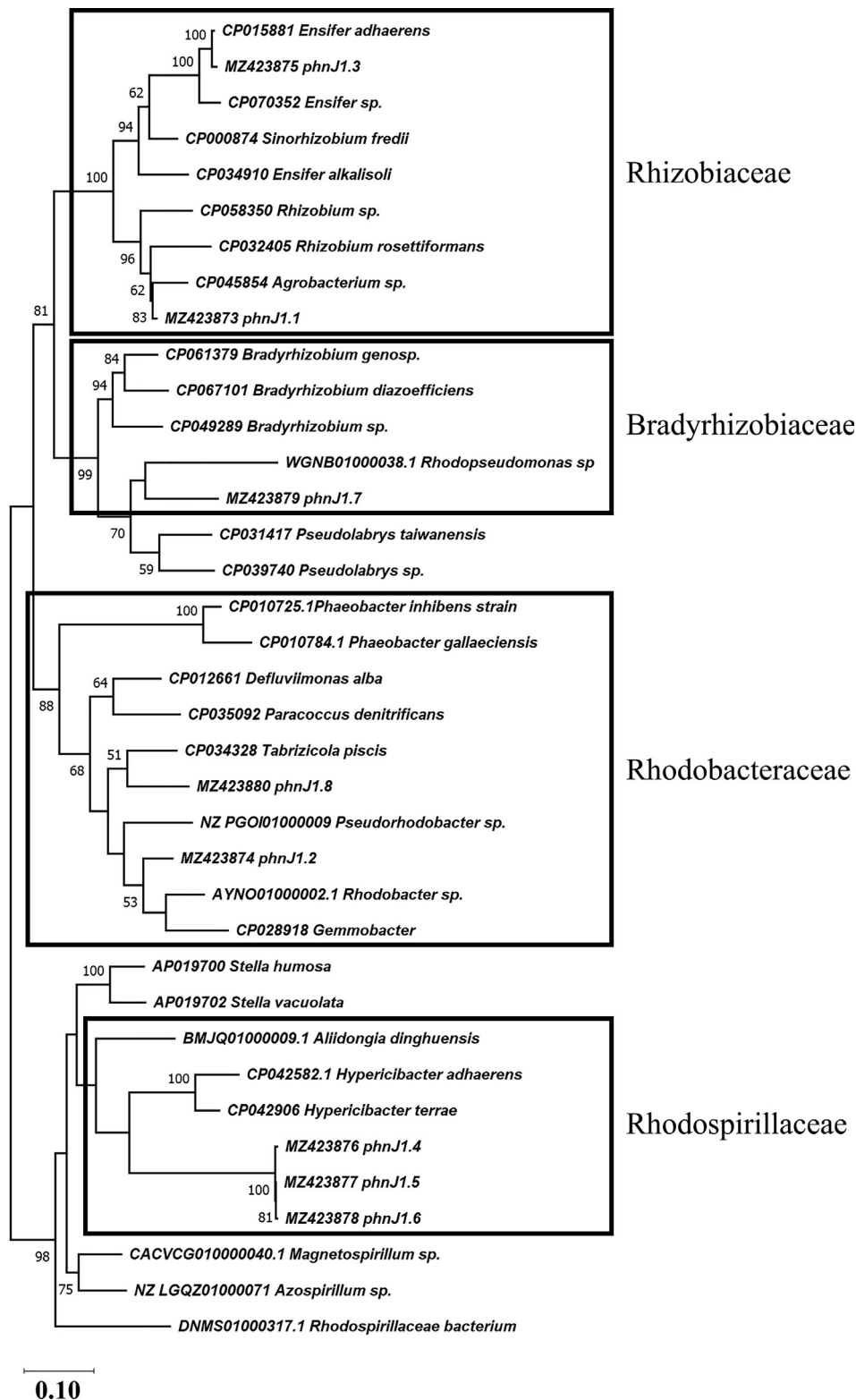

Fig. 2. Phylogram constructed using phnJ sequences amplified with Set 1 primers. The phylogram was constructed using the maximum-likelihood method: General Time Reversible with a gamma distribution (GTR + G). Numerical values at node branches indicate bootstrap values.

cies that belong to the families Rhodobacteraceae, Rhodospirillaceae, Rhyzobiaceae, and Bradyrhizobiaceae (Fig. 2). In the same way, the phylogram constructed with sequences obtained with Set 2 primers formed a group with *phnJ* genes related to species from Acetobacteraceae, Enterobacteraceae, Pseudomonadaceae, and Rhodobacteraceae (Fig. 3). The phylogenetic relationships of each set of sequences are different except for *phnJ1.2* and *phnJ2.12*, both sequences retrieved from Limoncocha Lagoon, which grouped with the same genera (*Rhodobacter* and *Gemmobacter*) in both pylograms.

To test whether the presence of these genes could actually translate into the isolation of functional glyphosate-degrading strains, Machángara sample was cultured in a medium with glyphosate as a unique carbon source. After the isolation procedure, three pure strains were obtained. These strains were also capable of growing using glyphosate as a unique phosphorous and nitrogenous source. According to its 16S RNA barcode sequence, these strains matched *Pseudomonas* sp. (100%), *Pantoea stewartii* (99.8%), and *Klebsiella varii* (100%). The *phnJ* gene from the isolates was amplified as a sequence. The *phnJ* sequence from *Pseudomonas*
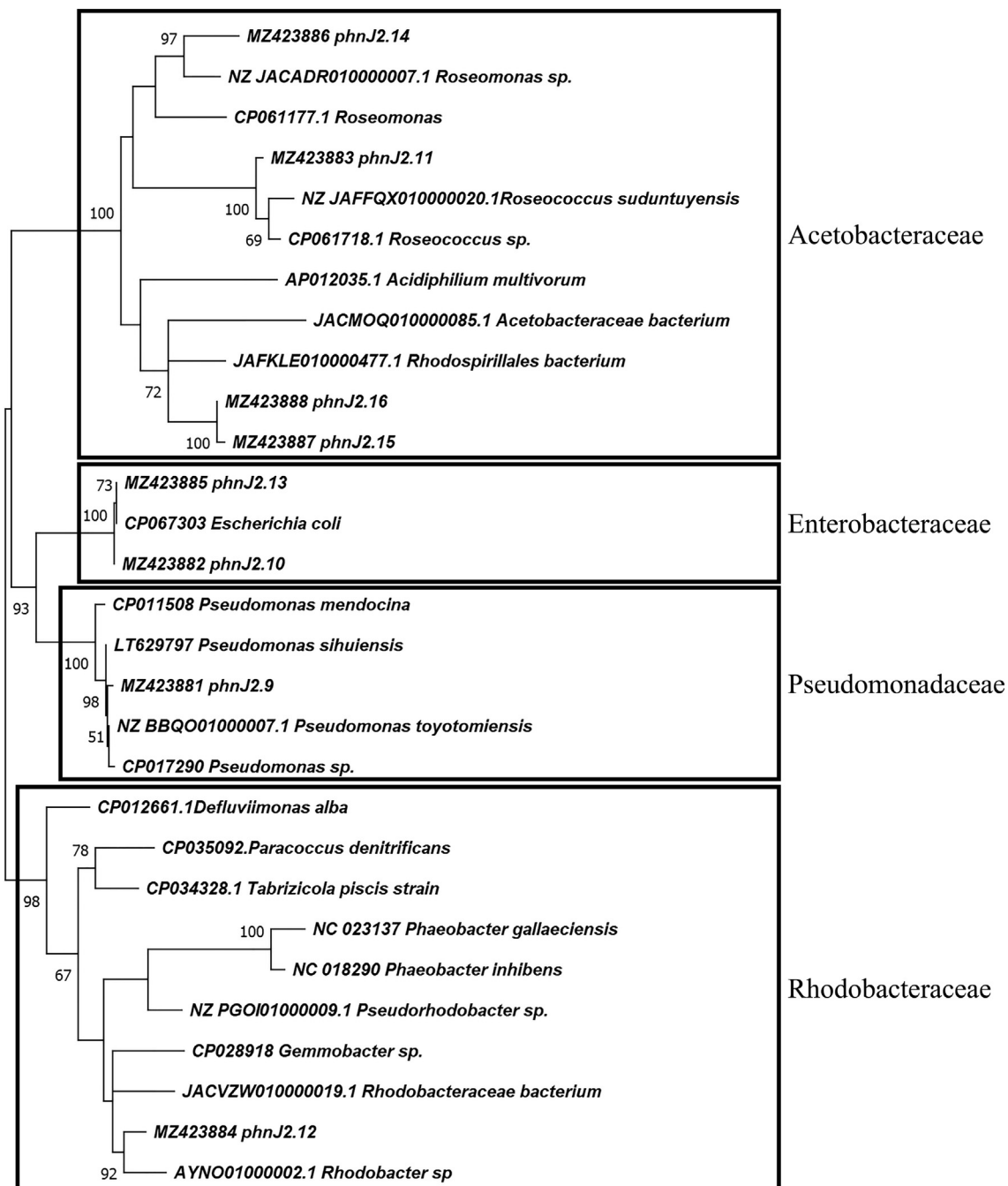

Fig. 3. Phylogram constructed using phnJ sequences amplified with Set 2 primers. The phylogram was constructed using the maximum-likelihood method: General Time Reversible with a gamma distribution (GTR + G). Numerical values at node branches indicate bootstrap values.

strain (*phnJ C.1*) and *Pantoea stewartii* strain (*phnJ* C.2) matched the enzyme sequence described for these species (100% and 99.5% identities, respectively). Nevertheless, *phnJ* from *K. variicola* strain (*phnJ* C.3) matched one assigned to *Enterobacter cloacae* with 96.8% identity. However, these three *phnJ* sequences were not detected in the eDNA analysis.

4. Discussion

Phosphonoacetaldehyde Hydrolase codified by *phnJ* gene is the second most abundant pathway for phosphonate degradation among sequenced bacteria (Villarreal-Chiu et al., 2012). C-P lyase operon has been subjected to gene insertions, substitutions, and deletion events so that it has great diversity either in specificity or functionality of the reaction and the sequences encoded (Villarreal-Chiu et al., 2012). Thus, it is important to retrieve new sequence encoding for these enzymes, including *phnJ*, which might have new properties for biotech industries.

Our results evidenced genetic potential for phosphonate degradation in all the aquatic systems assessed except the location in Numbayme River. This could be explained by the fact that this sampling site is surrounded by livestock areas, whereas the others are in agricultural areas, so it could be less affected by pesticide or herbicide contamination that has been associated with the presence of phosphonate-degrading microorganisms (Mauffrey et al., 2017).

We detected *phnJ* sequences of *Escherichia coli* in Yahuarcocha and Pita River, which might come from the urban sewage from Ibarra city and Quito metropolitan area respectively. This bacterium was first described by Zelenick et al. (1963) as an effective methyl phosphonate and ethylphosphonate biodegrader. The *phnj1.1* sequence is likely to belong to the *Rhizobium* genus (Fig. 2) since most *Agrobacterium* species have been reclassified as *Rhizobium* species (Farrand et al., 2003; Young et al., 2001). This genus has been broadly reported to degrade glyphosate, although the strains we found in the literature had been isolated from soil (Stosiek et al., 2020; I. P. Singh et al., 1999; K. Singh et al., 2021). In fact, genes from *A. tumefaciens* have been used to create glyphosate-resistant GMOs (Tian et al., 2015).

Species from the *Ensifer* genus, such as *E. meliloti* in flooded rice fields or *E. fredii* in soil from soya crops, have been described as resistant to glyphosate (Widowati & Ginting, 2020; Yang et al., 2020). *Ensifer* sp. might be an accidental host in Machángara River as it is a soil-dwelling bacterium. Thus, it most likely came from the riverbank soil or plant roots. *Pseudomonas* species are widely present in water, soil, plants, and animals, and have been identified as capable of carrying out the degradation process of both glyphosate and its metabolites (Singh et al., 2020; Solomon & Thompson, 2003). *phnj2.9* matched *Pseudomonas toyotoyensis,* whose complete genome has been sequenced from a strain present in date palm rhizosphere (Elmahi et al., 2021). *phnj2.11* matched *Roseococcus thiosulfatophilus* C-P lyase. We have not found any species from this genus reported as a glyphosate degrader in the literature, although there are several species from this genus with reported *phnJ* genes in the GenBank.

The phylogenetic analysis showed that the sequences found in the Ecuadorian water bodies are related to seven different families. This validates the usefulness of primers designed to screen for the presence of different types of phosphonate-degrader organisms in aquatic systems. Previous primers designed by Morales et al. (2020) proved able to detect and successfully quantify *phnJ* genes in soil; however, the amplicon size was shorter and the sequences obtained with these primers were related to species other than those detected in our work. Moreover, we detected the presence of what are probably new *phnJ* sequences, for example *phnJ1.4–6*

and *phnJ2.15–16*, which present a low similarity percentage with published sequences from the Rhodospirillales order. The variability found in some locations opens the door for future biodiversity studies, although the main objective of sequencing the amplicons was to confirm the presence of the gene of interest, not to perform a biodiversity study.

Furthermore, we have isolated three potential glyphosate-degrader strains, from the Machángara River sample. Nonetheless, the *phnJ* sequences from the isolates were different from those detected by eDNA analysis. This could be due to the low number of PCR clones analyzed. As mentioned before, this study did not set out to be a biodiversity assessment.

As aforementioned, several *Pseudomonas species* have been described as glyphosate degraders (Singh et al., 2020; Solomon & Thompson, 2003). *Klebsiella variicola* has been reported to be able to grow in glyphosate media as a carbon source, nevertheless, we found no records regarding *Pantoea stewartii* and glyphosate degradation in the literature Kurtoglu, et al., 2020). Besides, the *phnJ* gene amplified from *K. variicola* matched *phnJ* sequences from *E. cloacae* genome. This fact might be due to horizontal transference that is a successful mechanism for adaptation in phylogenetic-related species; such is the case for *Klebsiella* and *Enterobacter* genera (Ikuma and Gunsch, 2012). In fact, nowadays, plasmid-mediated bioaugmentation is a trending biorremediation strategy (Garbisu et al., 2017).

5. Conclusions

The effectiveness of the use of Nested PCR and primers designed to detect the presence of species with phosphonate degrading genes has been proven in eutrophicated Ecuadorian water bodies. Most of the amplified sequences are similar to genes from species described as phosphonate degraders. Five new sequences have been found that might belong to the order Rhodospirillales, three to order Rhodobacterales, and one to Hyphomicrobiales. Furthermore, three strains isolated from Machángara River have been proved to use glyphosate as a carbon, phosphorous, and nitrogen source, though they do not contain new gene variants. These results highlight the potential of Ecuador's ecosystems in the search for new species and/or genes that might have new properties for biotech industries, laying the groundwork for additional research. Further studies are necessary to address the isolation and characterization of these new enzymes.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2021.11.013.

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