



# Diversity and enzymatic potentialities of *Bacillus* sp. strains isolated from a polluted freshwater ecosystem in Cuba

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## Abstract

Genotypic and phenotypic characterization of *Bacillus* spp. from polluted freshwater has been poorly addressed. The objective of this research was to determine the diversity and enzymatic potentialities of *Bacillus* spp. strains isolated from the Almendares River. *Bacilli* strains from a polluted river were characterized by considering the production of extracellular enzymes using API ZYM. 14 strains were selected and identified using 16S rRNA, *gyrB* and *aroE* genes. Genotypic diversity of the *Bacillus* spp. strains was evaluated using pulsed field gel electrophoresis. Furthermore, the presence of genetic determinants of potential virulence toxins of the *Bacillus cereus* group and proteinaceous crystal inclusions of *Bacillus thuringiensis* was determined. 10 strains were identified as *B. thuringiensis*, two as *Bacillus megaterium*, one as *Bacillus pumilus* and one as *Bacillus subtilis*. Most strains produced proteases, amylases, phosphatases, esterases, aminopeptidases and glucanases, which reflect the abundance of biopolymeric matter in Almendares River. Comparison of the typing results revealed a spatio-temporal distribution among *B. thuringiensis* strains along the river. The results of the present study highlight the genotypic and phenotypic diversity of *Bacillus* spp. strains from a polluted river, which contributes to the knowledge of genetic diversity of *Bacilli* from tropical polluted freshwater ecosystems.

**Keywords** *Bacillus* spp. · *Bacillus thuringiensis* · Enzyme activity · Freshwater · Polluted river

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## Introduction

Microorganisms play important roles in all ecosystems, contributing to almost half of the global primary productivity and driving major biogeochemical cycles (Litchman 2010; García-Armisen et al. 2014; Mandic-Mulec et al. 2015). In aquatic ecosystems, bacteria degrade and mineralize organic compounds, passing the reserves of dissolved organic matter to higher trophic levels (Huang et al. 2011; Peter 2011; Castelo-Branco et al. 2016). In this context, important metabolic functions, including degradation of organic matter and assimilation of carbon, nitrogen and phosphorus are catalyzed by enzymes produced by microorganisms (Frossard 2011; Yuan et al. 2015; Fabian et al. 2017).

Among bacteria, *Bacillus* spp. are producers of specific extracellular enzymes, such as proteases, amylases, phosphatases, lipases or esterases. These enzymes are important in biogeochemical cycles of nutrients and also have potential industrial and biotechnological applications. Indeed, it has been suggested that these endospore forming bacteria can

contribute to the self-purification of water and therefore may have greater biotechnological potential compared to other bacterial groups (Bal et al. 2009; Geethanjali and Subash 2011; Mandic-Mulec et al. 2015; Mohammad et al. 2017). In particular, the ability of certain *Bacillus* spp. to produce extracellular enzymes in aquatic systems plays an important role in the decomposition of biopolymers, including proteins, cellulose and other complex carbohydrates.

Moreover, it has been observed that bacterial communities associated with the roots of aquatic macrophytes are key players in recycling of nutrients in aquatic ecosystems. For instance, heterotrophic and epiphytic bacteria from several macrophytes such as *Eichhornia crassipes* (water hyacinth), *Nymphaea lotus* (Egyptian water lily) and *Pistia stratiotes* (water lettuce) can reduce the rate of eutrophication (Haroon and Daboor 2009; Moyo et al. 2013; Yan et al. 2016; Kumar et al. 2017). Yi et al. (2013) suggested that the cultivation of *E. crassipes* in eutrophic waters can contribute to increase the diversity and abundance of denitrifying bacteria, resulting in a greater release of nitrogen gas through the denitrification process. For this reason, the study of microorganisms that inhabit the roots of aquatic plants can contribute to the recovery of contaminated aquatic ecosystems.

The Almendares River is a polluted ecosystem located in a densely populated area of La Habana, Cuba. This water body receives large amounts of organic matter in the form of domestic and industrial wastes, which are responsible for its eutrophic state (Arpajón et al. 2011), including high heavy metal levels and other contaminants in its sediments (Olivares-Rieumont et al. 2005; Knapp et al. 2012). This is of local concern because the Almendares watershed provides over 47% of La Habana water supply (via groundwater) and contains one of the largest urban parks in Cuba (Arpajón et al. 2011; Graham et al. 2011). This ecosystem has been previously evaluated taking into account microbial and chemical indicators (Arpajón et al. 2011; Romeu et al. 2015). However, little is known about the *Bacillus* spp. diversity and their potentiality as depollution agent for freshwater ecosystems like the Almendares River. Therefore the objective of this research was to determine the diversity and enzymatic potentialities of *Bacillus* spp. strains isolated from the Almendares River.

## Materials and methods

### Sites and sample collection

The samples were collected from three sampling stations in the Almendares River (Río Cristal, Paila and Puente de Hierro) on February, April, June and October 2009. This river located in western part of Cuba, is approximately 45 km in surface area and serves as one of the primary

drinking water resource to the Havana metropolitan area. All the sampling stations showed a high degree of eutrophication (Arpajón et al. 2011). Río Cristal (RC) is an upstream station with abundant macrophytes such as *E. crassipes* (water lilies) and *P. stratiotes* (water lettuce). The drainage area is characterized by a high proportion of agricultural and cattle lands, which results in a high-nutrient load to the system. Paila (P) is an affluent of the Almendares River and is the most contaminated station with the highest values of thermotolerant coliforms and inorganic nutrients like ammonium, nitrate, nitrite and phosphate (Arpajón et al. 2011; Romeu et al. 2015). The drainage area is characterized by a high proportion of urban areas and the presence of a pharmaceutical laboratory. Puente de Hierro (PH) is located at the river mouth and presents high salinity values; this estuary is also heavily contaminated.

Water samples were collected in 2L sterile plastic bottles placed in a chilled cooler. Plant samples were selected according to the most abundant species at each location. One quadrant of 10 × 10 m at the left riverbank was measured, and the plants located in the corner and in the center of the quadrant were collected (five plants for each species). The selected plants were *E. crassipes* (RC and PH), *P. stratiotes* (RC), *Cabomba* sp. (RC), *Commelina* sp. (RC and P), *Bidens pilosa* L. (RC, P and PH) and *Hydrocotyle* sp. (RC and P). All samples were collected in the morning and processed within 4 h. During the sampling period, the river flow remained low and some of the plants were growing in places originally occupied by river flows.

### Isolation from water and rhizosediment

Samples from water were serially diluted from  $10^{-1}$  to  $10^{-6}$  in saline solution (NaCl, 0.8%) and spread in Nutrient Agar; 0.1 mL of each dilution was plated in triplicates. Plates were incubated at 30 °C, for 48–72 h. After incubation, representative colony forming units (CFU) displaying different morphologies were isolated and re-streaked on Nutrient Agar plates until purification.

For rhizosediment samples, five plants from the same species in each sampling station were collected and homogenized. The roots with the adhered sediment (rhizosediment) were cut and one gram was diluted in 100 mL of saline solution. Serial dilutions were performed from  $10^{-1}$  to  $10^{-6}$  in saline solution and spread in Nutrient Agar. The rhizosediment samples followed the same protocol as the water samples.

To determine the presence of extracellular enzymes, the most frequent bacterial groups from the three sampling stations and the different Almendares River plants, were selected based on their morphological and staining (Gram test) characterization.

## Determination of protease and amylase activities

For each isolate, a bacterial suspension of ca.  $1 \times 10^8$  CFU mL<sup>-1</sup> (0.5 Mc Farland unit) was prepared. To determine the proteolytic potential, bacteria were grown in Tryptone Soy Agar (TSA) containing gelatin 1% and incubated at 37 °C for 48 h. The plates were then covered with 8–10 mL of Frazier reagent. The non-hydrolyzed gelatin forms a white precipitate with the reagent while hydrolyzed gelatin appears as a clear zone. The enzyme production was assessed as the difference between the diameter (in mm) of the colony and the diameter of the halo (Gorlach-Lira and Coutinho 2007).

To evaluate their amylolytic potential, bacteria were grown in TSA containing 1% of soluble starch and incubated at 37 °C for 48 h. 5 mL of iodine solution (1%) was added to the plate and the presence of a colorless halo around the colony indicated amylase secretion. The enzyme production was assessed as the difference between the diameter (in mm) of the colony and the diameter of the halo (Khan et al. 2011).

## APY ZYM system assay for enzymatic activity determination

The evaluation of extracellular enzymes production with API ZYM (Biomérieux, France) was carried out with the best isolates in proteases and amylases production. The strips were inoculated following the manufacturer's instructions and incubated for 4 h and 30 min at 37 °C. After incubation, one drop of ZYM A reagent and one drop of ZYM B reagent were added to each microcupule. Color development was scored as positive and no color development as negative. For microcupule that showed positive results, a value ranging from 1 to 5 (1 = 5 nM; 2 = 10 nM; 3 = 20 nM; 4 = 30 nM; 5 = 40 nM) was assigned according to the color chart provided by the manufacturer.

## DNA extraction, PCR amplification and gene sequencing

Bacterial DNA was extracted using the SDS-phenol-chloroform method (Eichner et al. 1999). The PCR amplification of the 16S rRNA gene was performed using 50 µL of the reaction mixture, containing 25 µL of PCR Master (Roche Diagnostics GmbH, Germany), a final concentration of 0.5 µM of each universal primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GTTACCTTGTACGACTT-3') (Zhou et al. 2009), 5 µL of template DNA and 15 µL of Milli Q water. After an initial 5-min denaturation step at 94 °C, samples were amplified during 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 90 s at 72 °C. The last cycle was followed by a final 5 min elongation at 72 °C.

The *gyrB* gene was amplified with the primer pair *gyrB*-F2 (5'-ACWCGTATGCGTGARYTRGC-3') and *gyrB*-R2

(5'-CCTTGYTTTGCWGAWCCDCC-3') (Soufiane and Côté 2009) and the primer pair *gyrF* (5'-AATAATAACTTTATGATAGCGT-3') and *gyrR* (5'-CGGTGGCGGTTACAAAGTTTC-3') (Swiecicka et al. 2008) for the strains belonging to the *Bacillus cereus* group. After an initial 5-min denaturation step at 94 °C, DNA was amplified for 30 cycles at a denaturing temperature of 94 °C for 30 s, annealing at 51 °C for 30 s, extension at 68 °C for 30 s and a final extension at 68 °C for 7 min. The *aroE* gene was amplified with primer pair *aroE* F1/R1: *aroE* F1 (5'-ATCGGAAATCCAATTGGACA-3') and *aroE* R1 (5'-CCTGTCCACATTTCAAAYGC-3') (Soufiane and Côté 2009) for the strains belonging to the *B. cereus* group. DNA was amplified for 30 cycles at denaturing temperature of 95 °C for 30 s, annealing at 47 °C for 30 s and extension at 72 °C for 60 s.

The amplified PCR fragments of the 16S rRNA, *gyrB* and *aroE* genes were sequenced, aligned and analyzed using both NCBI GenBank (<http://www.ncbi.nlm.nih.gov/BLAST>) (Altschul et al. 1990) and Ribosomal Database Project (<http://rdp.cme.msu.edu>) (Cole et al. 2009). The bootstrap consensus tree inferred from 1000 replicates was used to construct the dendrogram of similarity. Phylogenetic analyses were conducted in MEGA5 (Tamura et al. 2011). Sequence data from this study were deposited in GenBank under the accession number KF910733, KF910744, KF910745, KF910748, KF910755–KF910764, KY113140–KY113149 and KY283086–KY283095.

## Evaluation of proteinaceous crystal production

To discriminate *Bacillus thuringiensis* among members of the *B. cereus* group, the presence of proteinaceous crystalline inclusions were determined using the method described by Santana et al. (2008).

## Pulse field gel electrophoresis (PFGE) of chromosomal DNA of *Bacillus* sp.

The low-melting-point agarose (Sigma Chemical Co., St Louis, MO) embedded chromosomal DNA was prepared from 6 h cultures according to Gaviria-Riviera and Priest (2003). The plugs were digested with 30 U of *NotI* (MBI Fermentas, Vilnius, Lithuania) in 250 µL of the buffer following the manufacturer's protocol for 16 h at 37 °C. PFGE was performed with the CHEF-DR II System (Bio-Rad Laboratories, Hercules, CA) at 14 °C and 5.6 V cm<sup>-1</sup>, using 1.0% (w/v) agarose gel in 0.5× TBE (100 mM Tris, 100 mM borate, 200 mM EDTA). The electrophoresis was carried out for 26 h, with a pulse from 3 to 90 s. After staining with ethidium bromide solution (1 µg mL<sup>-1</sup>) the gels were analyzed with the BioCaptMW version 99.05 s system (Vilber Lourmat, Marine-La-Vallee, France). Yeast Chromosome PFG Marker and Lambda Ladder PFG Marker (New

England BioLabs, Massachusetts, USA) were used as mass markers according to Swiecicka and Mahillon (2006). The PFGE pattern was analyzed using Gel Compare II version 6.0 (Applied Maths, Sint-Martens-Latem, Belgium).

### Presence of virulence genetic determinants of *B. cereus* group

The detection of virulence genetic determinants was performed by PCR using the primers and thermocycling parameters described in Online Resource 1. PCR amplifications were carried out using a C1000 Thermal Cycler (Biorad, Winninglaan, Temse). Final reaction mixtures (20 µL) contained approximately 70 ng total DNA template, 1× PCR buffer (Promega 5× Green GoTaq Reaction Buffer), 200 µM of each dNTP, 1.5 µM of MgCl<sub>2</sub>, 1 µM each of forward and reverse primers and 0.02 units GoTaq DNA polymerase (Promega, Madison, WI, USA). PCR products were analyzed by electrophoresis in 1% agarose gel. *B. cereus* strain Kinrooi 5975c (Dierick et al. 2005) was included as a reference in the PCR assays to detect the virulence genetic determinants *nheA*, *clo*, *hbl*, *eml*, *ces* and *cer*. The genetic determinants *nheA*, *clo*, *hbl*, correspond to the subunit A of non-hemolytic enterotoxin, cerolysin O and hemolysin BL, respectively. The last three virulence genetic determinants (*eml*, *ces* and *cer*) correspond to emetic toxin synthesis-related genes. In the case of *cytK1* and *cytK2*, coding for the two variants of cytolysin K (CytK), *Bacillus cytotoxicus* LMG 26718<sup>T</sup> was used as a reference strain for *cytK1*, while strain *B. cereus* strain Kinrooi 5975c was used as control for *cytK2*. SmartLadder (Eurogentec, Liège, Belgium) was used as a molecular mass marker. Gels were analyzed with the BioCaptMW version 99.05 s system (Vilber Lourmat, Marine-La-Vallee, France).

## Results

### Morphological characterization of bacterial isolates

Based on morphological and staining characteristics, bacterial isolates of the Almendares River were classified into four groups as shown in Table 1. Gram-positive rod-shaped endospore-forming bacteria predominated in both water and rhizosediment samples. From the total of Gram-positive rod-shape endospore-forming bacteria, 64 isolates (42 from rhizosediment and 22 from water samples) were selected for the evaluation of their extracellular enzyme activities.

### Extracellular enzyme activity

The production diameters of proteases and amylases obtained from plant rhizosediment and water isolates

**Table 1** Distribution of water and rhizosediment bacterial isolates from the Almendares River

| Bacterial group               | Water isolates | Rhizosediment isolates |
|-------------------------------|----------------|------------------------|
| Gram-positive                 |                |                        |
| Cocci                         | 10             | 18                     |
| Rod-shaped, endospore-forming | 32             | 101                    |
| Rod-shaped, non-sporulating   | 8              | 16                     |
| Gram-negative                 |                |                        |
| Rod-shaped                    | 10             | 50                     |
| Total                         | 60             | 185                    |

respectively, are shown in Online Resource 2 and Online Resource 3. From the 64 isolates, 60 displayed protease activity and 48 showed amylase activity. The 36 best protease and amylase producers (24 from rhizosediment and 12 from water samples) were retained for the determination of others enzymes production by API ZYM.

Distinct profiles of extracellular enzyme activity were observed in water isolates. Alkaline phosphatase, esterase (C 4), esterase lipase (C 8), naphthol-AS-BI-phosphohydrolase, acid phosphatase, α-glucosidase, leucine aminopeptidase, valine aminopeptidase α-chymotrypsin and β-glucosidase were the enzymes more frequently detected (Fig. 1). None of the water bacteria showed evidence of activity of β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase or α-fucosidase. Enzymatic activity profiles of water isolates are shown in Online Resource 4.

In rhizosediment isolates, esterase (C 4), esterase lipase (C 8), naphthol-AS-BI-phosphohydrolase, alkaline phosphatase, acid phosphatase, valine aminopeptidase, leucine aminopeptidase, β-glucosidase and α-glucosidase were the enzymes more frequently detected (Fig. 2), and none of the rhizosediment isolates showed evidence of β-glucuronidase, α-mannosidase or α-fucosidase activity. Enzymatic activity profiles of rhizosediment isolates are shown in Online Resource 5.

Based on the production of extracellular enzymes (Online Resource 6), the representation of the different sampling stations and collected plants of the Almendares River and their different micro-morphological and cultural characteristics, 14 isolates (9 from rhizosediment and 5 from water samples) were selected for further analysis.

### Identification of *Bacillus* spp. strains

The molecular identification of the strains is shown in Online Resource 7. BLAST analysis of the 16S rRNA sequences indicated that 10 strains belong to the *B. cereus* group, two to *B. megaterium*, one to *B. pumilus* and one to *B. subtilis*.

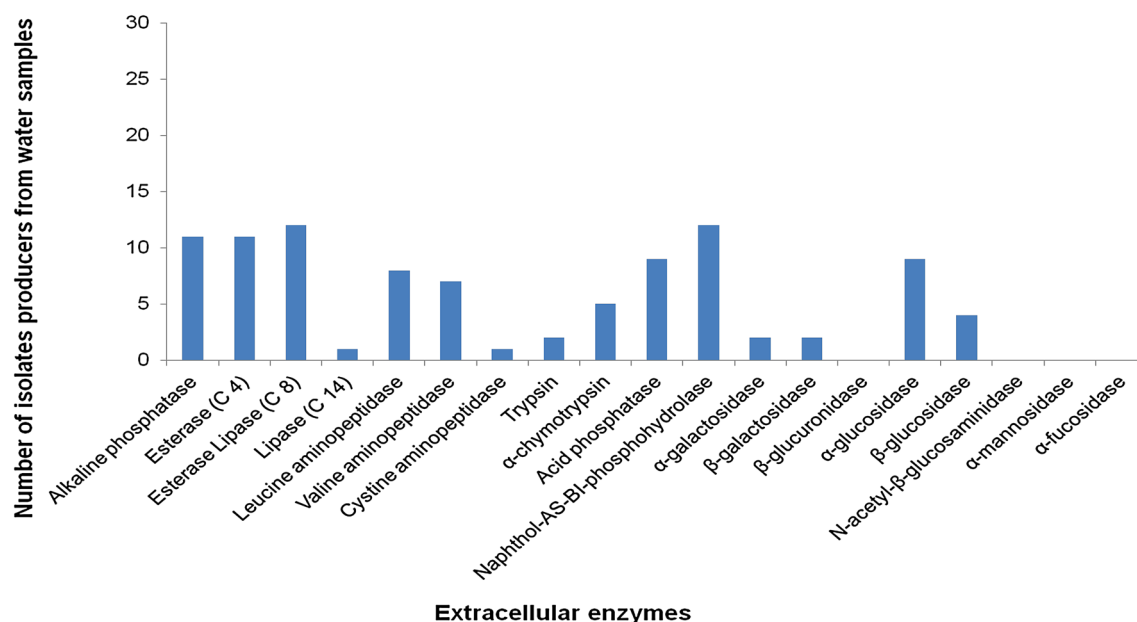


Fig. 1 Number of extracellular enzyme producers from water samples

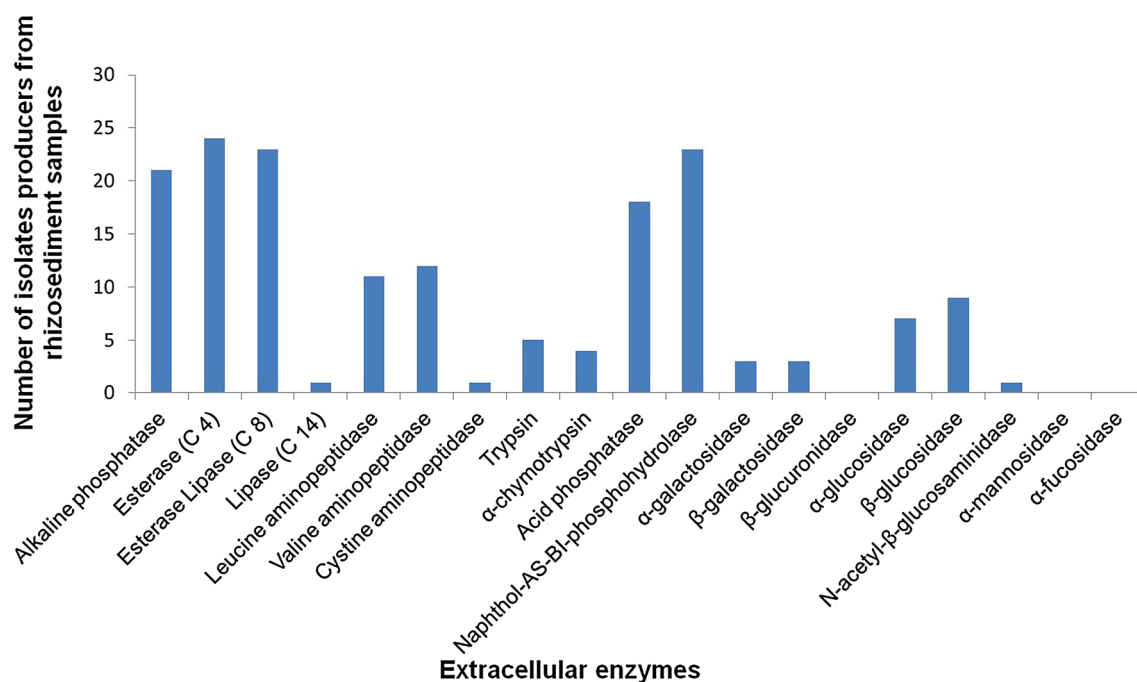
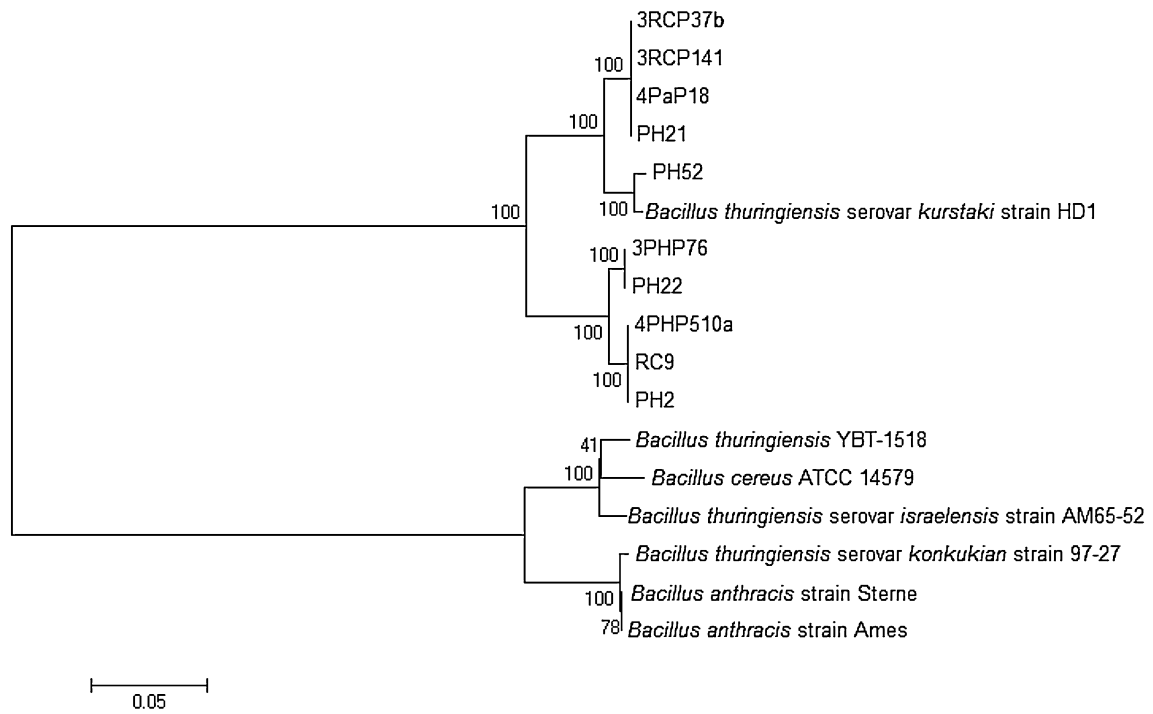


Fig. 2 Number of extracellular enzyme producers from rhizosediment samples

Further discrimination between the ten *B. cereus s.l.* strains was obtained by sequencing and comparing their *gyrB* and *aroE* genes. Bootstrapped neighbor-joining trees were constructed based on the aligned sequences. The best discrimination among the strains was obtained with the *aroE* gene, as shown in Fig. 3. In this case, the analysis showed two groups at 95% nucleotide sequence identities,

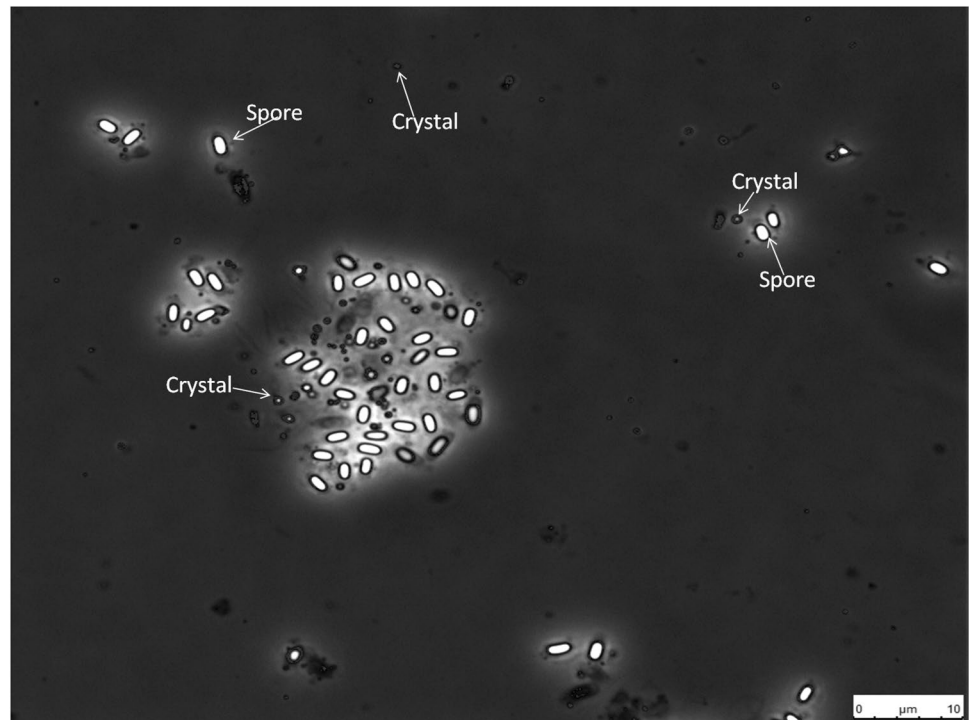
where the 10 *B. cereus sensu lato* strains clustered with *B. thuringiensis* serovar *kurstaki* strain HD1, as compared to a group of reference strains.

As shown in Fig. 4, the presence of crystalline inclusions in sporulated cultures of the 10 *B. cereus sensu lato* strains identified them as *B. thuringiensis*.



**Fig. 3** Bootstrapped neighbour-joining trees of *B. cereus* group strains generated from the alignment of *aroE* nucleotide sequences. Bootstrap values higher than 50% are indicated. The horizontal bar represents 0.05% differences in nucleotide identities

**Fig. 4** Crystal and spores of strain 4PHP510a under a phase contrast microscope at 1000 fold magnification



## Analysis of the diversity of *Bacillus* spp. strains by PFGE

The diversity of the 14 *Bacillus* spp. strains was also characterized by PFGE analysis. The genomic DNA of the *Bacillus* spp. strains was analyzed with *NotI* restriction enzyme, which yielded three to five fragments within the *B. thuringiensis* strains, nine different fragments for *B. subtilis*, 11 for *B. pumilus* and 12 for *B. megaterium* PailaP1c (Fig. 5). Using a similarity threshold of 70%, a total of eight distinct pulsotypes were observed. Particularly, within the *B. thuringiensis* strains, four pulsotypes were observed (clusters A to C, and the singleton PH52). Moreover, all the strains of cluster A and cluster C, as well as the strains PH2 and RC9 from cluster B, could not be differentiated, which suggests the possible clonality of the corresponding strains. Interestingly, the strains pertaining to the same clusters were obtained during the same sampling dates (with the exception of 4PaP18 in cluster C); clusters B and C included strains isolated from different sampling stations.

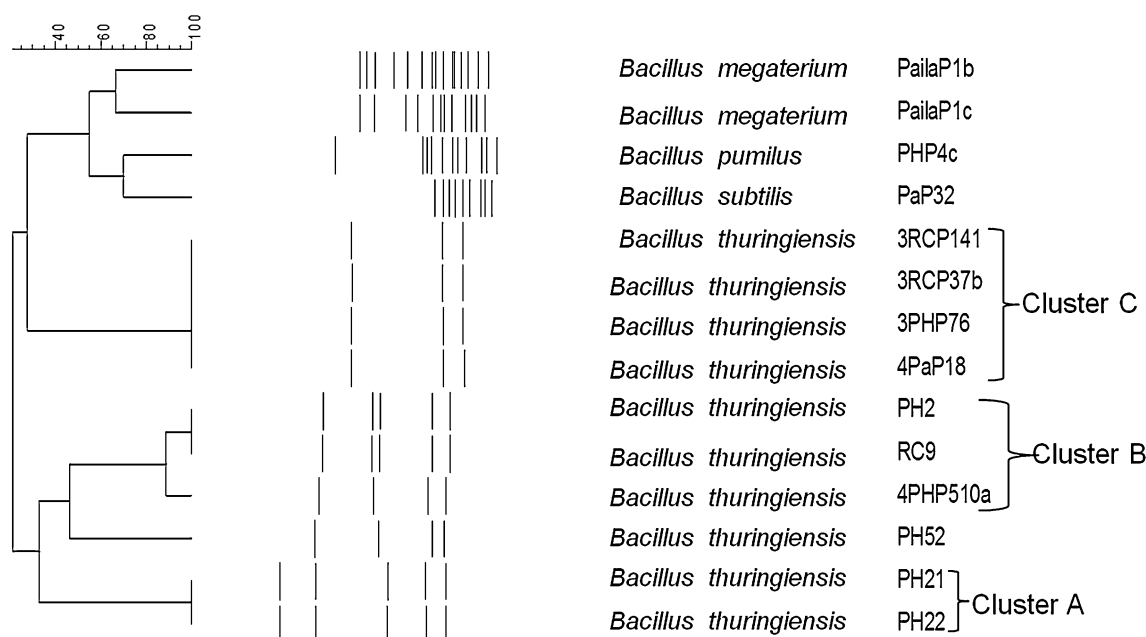
## Detection of genetic determinants of potential virulence toxins

Determination of genetic determinants of potential virulence toxins is important to analyze the pathogenicity of the strains isolated from a polluted river. As shown in Table 2, detection of the genetic determinants of cytotoxin K, cereolysin O, non-hemolytic enterotoxin, the emetic toxin (cereulide) and

hemolysin BL using PCR, demonstrated that all the strains identified as *B. thuringiensis* were positive for *cytK2*, eight for *clo* and five for *nheA*. None were positive for *cytK1*, *hbl* or the cereulide genetic determinants. The strains of *B. megaterium*, *B. pumilus* and *B. subtilis* did not display any positive PCR reaction suggesting the absence of the corresponding virulence factors. The occurrence patterns of the potential virulence factors matched well with particular pulsotypes (Fig. 5). The only difference among the 4 strains of cluster C was the presence of *nheA* gene in the 3PHP76 and 4PaP18 strains, while it was absent in the other 2 strains.

## Discussion

Microbiota composition varies as a function of water type and depends mainly on salt and organic compound concentration, turbidity, temperature, and contamination sources (Reche and Fiuza 2005; Herfort et al. 2017). In aquatic ecosystems, the most common bacteria are Gram-negative rods (Ramkumar et al. 2011). However, in polluted ecosystems, endospore-forming bacteria have advantages over others bacteria; when conditions are not optimal, endospore formation is triggered. Spores are resistant structures intended to protect and conserve the genetic material of the organisms until the conditions become suitable for vegetative growth (Bueche et al. 2013). In the present study, endospore-forming bacteria were predominant among water and rhizosediment samples of the Almendares River (a polluted



**Fig. 5** Dendrogram of *Bacillus* spp. strains from Almendares River characterized by PFGE typing using *NotI* as restriction enzyme. Clusters were formed at 70% of similarity

**Table 2** Detection of the genetic determinants of potential virulence toxins in *Bacillus* spp. strains of the Almendares River

| Strains  | Cluster <sup>a</sup> | Identification          | Virulence genetic determinants <sup>b</sup> |              |            |             |            |            |            |            |
|----------|----------------------|-------------------------|---------------------------------------------|--------------|------------|-------------|------------|------------|------------|------------|
|          |                      |                         | <i>cytK1</i>                                | <i>cytK2</i> | <i>clo</i> | <i>nheA</i> | <i>eml</i> | <i>ces</i> | <i>cer</i> | <i>hbl</i> |
| PH21     | A                    | <i>B. thuringiensis</i> | —                                           | +            | —          | +           | —          | —          | —          | —          |
| PH22     | A                    | <i>B. thuringiensis</i> | —                                           | +            | —          | +           | —          | —          | —          | —          |
| PH2      | B                    | <i>B. thuringiensis</i> | —                                           | +            | +          | —           | —          | —          | —          | —          |
| RC9      | B                    | <i>B. thuringiensis</i> | —                                           | +            | +          | —           | —          | —          | —          | —          |
| 4PHP510a | B                    | <i>B. thuringiensis</i> | —                                           | +            | +          | —           | —          | —          | —          | —          |
| 3RCP141  | C                    | <i>B. thuringiensis</i> | —                                           | +            | +          | —           | —          | —          | —          | —          |
| 3RCP37b  | C                    | <i>B. thuringiensis</i> | —                                           | +            | +          | —           | —          | —          | —          | —          |
| 3PHP76   | C                    | <i>B. thuringiensis</i> | —                                           | +            | +          | +           | —          | —          | —          | —          |
| 4PaP18   | C                    | <i>B. thuringiensis</i> | —                                           | +            | +          | +           | —          | —          | —          | —          |
| PH52     | —                    | <i>B. thuringiensis</i> | —                                           | +            | +          | +           | —          | —          | —          | —          |
| PailaP1b | —                    | <i>B. megaterium</i>    | —                                           | —            | —          | —           | —          | —          | —          | —          |
| PailaP1c | —                    | <i>B. megaterium</i>    | —                                           | —            | —          | —           | —          | —          | —          | —          |
| PHP4c    | —                    | <i>B. pumilus</i>       | —                                           | —            | —          | —           | —          | —          | —          | —          |
| PaP32    | —                    | <i>B. subtilis</i>      | —                                           | —            | —          | —           | —          | —          | —          | —          |

<sup>a</sup>Cluster refers to the agglomeration in Fig. 5

<sup>b</sup>*CytK1* and *cytK2* encode the cytotoxin K variants, *clo* encodes cereolysin O, *nheA* encodes subunit A of non-hemolytic enterotoxin, *hbl* encodes hemolysin BL and *eml*, *ces*, *cer* are fragments of emetic toxin synthesis-related genes

ecosystem with high concentrations of organic matter from domestic and industrial wastewater). These observations confirmed those made by Reche and Fiuza (2005) reporting that in aquatic systems polluted with organic compounds and non-treated domestic effluent, Gram-positive bacteria predominate in water and sediments when a culture-dependent approach is used.

The 36 isolates were selected as representative bacteria of the three sample stations of the Almendares River as well as from the different plants collected. For the rhizosediment isolates, although previous isolations from *E. crassipes* and *P. stratiotes* have been made (Haroon and Daboor 2009), no previous isolation from *Commelina* sp., *Hydrocotyle* sp., *Cabomba* sp. and *B. pilosa* L have been reported in Cuba. Association between these microorganisms and their aquatic macrophytes could favor phytoremediation processes due to the potentialities of microorganisms in nutrients solubilizing and plant growth promoting as was suggested by Li and Wong (2011).

The extracellular enzyme activity of the isolates was assessed using API ZYM. The production of phosphatases, esterases, aminopeptidases and glycosyl hydrolases by endospore-forming rods showed the metabolic capabilities associated with the Almendares River isolates and is indicative of the polymeric composition of the sources of organic matter, as previously suggested by Mudryk and Skórczewski (2006).

Phosphatases are the group of phosphohydrolases that most intensively participate in phosphate release in aquatic environments (Cunha et al. 2010). Phosphatases can be

present in two forms: alkaline and acid, both able to hydrolyse phosphoric esters (Mudryk and Skórczewski 2006). In the present work, the majority of the endospore-forming rods, identified as *Bacillus* species, produced from one to three phosphatases. This observation is in agreement with Mudryk and Podgórska (2006), which reported phosphatase-producing capacity in several heterotrophic bacterial genera like *Pseudomonas*, *Chromobacterium*, *Bacillus* or *Flavobacterium*.

Esterases were also one of the most active extracellular enzymes detected in Almendares River isolates. The isolates showed higher lipolytic activity against short-chain fatty acids, than against lipids with long-chain fatty acids. Other enzymes such as leucine aminopeptidase and valine aminopeptidase were also frequently found among the freshwater isolates. Leucine aminopeptidase is mainly associated with heterotrophic bacteria and hydrolyses a large number of peptides and aminoacid amide of the L-configuration, with particular affinity to L-leucyl-peptides and L-leucyl-amides (Cunha et al. 2010). It has been suggested that the level of leucine aminopeptidase is a good measure of the proteolytic activity of bacteria (Mudryk and Skórczewski 2006), something that was noticed in this work by the evaluation of proteases production. Finally, glycosyl hydrolase activities were present in some of the rhizosediment and water isolates. Tiquia (2011) suggested that the presence of these enzymes is consistent with degradation of plant, animal and microbial detritus. Therefore, these isolates could contribute to decaying matter degradation in the Almendares River, participating in carbon recycling to the biosphere.

The *B. cereus s.l.* complex comprises seven closely related species: *B. cereus sensu stricto*, *B. anthracis*, *B. thuringiensis*, *B. weihenstephanensis*, *B. mycoides*, *B. pseudomycoides* and *B. cytotoxicus* (Guinebretière et al. 2013), all sharing 16S rRNA sequence identity higher than 99% (Guinebretière et al. 2010; Martínez-Blanch et al. 2011; Maughan and Van der Auwera 2011; Økstad and Kolstø 2011; Guinebretière et al. 2017). However, since *B. thuringiensis* can be distinguished from the rest of the group by its ability to produce crystal protein inclusions (crystals) during sporulation (Aronson 2002; Santana et al. 2008), the 10 strains belonging to the group *B. cereus s.l.* could be identified as *B. thuringiensis* (Fig. 4, Online Resource 7).

*B. thuringiensis* is an entomopathogenic bacterium used as biopesticide because its toxins are harmless to humans and other mammals, birds, amphibians, reptiles and plants and are biodegradable, thus no residual toxic product accumulates in the environment (Bravo et al. 2011; Schneider et al. 2017). This bacterium has therefore been used worldwide for the selective control of mosquito larvae populations (Despres et al. 2011; Fayolle et al. 2015; Schneider et al. 2017). The diversity of the strains tested by PFGE in the present study showed that *B. thuringiensis* strains were heterogeneous, as was previously reported for this species (Swiecicka et al. 2013; Chen et al. 2014). Comparison of the PFGE typing and the profile of virulence genetic determinants showed only one difference in cluster C, where *nheA* gene was present in two of the strains of the group but absent in the other two.

According to the spatial distribution of *B. thuringiensis* clones, all the strains from Río Cristal station harbored the virulence genes *cytK2* and *clo* (3RCP141, 3RCP37b and RC9). These virulence genes were present also in the strains PH2 (clone of RC9) and 4PHP510a (similar to RC9) which came from Puente de Hierro station (10 km downstream from Río Cristal). Hence, this could indicate that some of the strains present in Puente de Hierro station came from the upstream station Río Cristal. Similar results were reported by Rajabi et al. (2011), who analyzed the genetic diversity of *Salmonella enterica* by PFGE in the Upper Suwannee River, and detected that two genogroups containing 39 and 35 isolates respectively were found in different locations of the river. Casarez et al. (2007) using enterobacterial repetitive intergenic consensus sequence polymerase chain reaction (ERIC-PCR) found the same *Escherichia coli* genotypes in two watersheds separated by 30 km, indicating some level of geographic stability of the *E. coli* populations from water. The present results suggest a spatial stability of some strains in the main course of the Almendares River.

In the case of the temporal distribution of the *B. thuringiensis* clones, the clusters showed a separation among the sampling collection date. Cluster A were formed by strains collected on February 2009, cluster B include strains from

October 2009 and cluster C was represented by strains isolated on June 2009, with the exception of 4PaP18 collected on October 2009. These observations may be indicative of seasonality in the occurrence of the *B. thuringiensis* strains. Similar results were reported by Casarez et al. (2007) and Rajabi et al. (2011) who detected a temporal variability of *E. coli* and *Salmonella enterica*, respectively, in freshwater sources.

In conclusion, the present study showed the genotypic and phenotypic diversity among *Bacillus* spp. strains isolated from a polluted river. By the use of microbial source tracking, it was possible to observe the dispersal of the strains across the main stream of the river. However, further analyses to evaluate agricultural input, rainfall, flow rates and other environmental parameters, that may influence microbial diversity of *Bacillus* spp. strains in polluted freshwater, are needed.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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