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Water-related environments: a multistep procedure to assess the diversity and enzymatic properties of cultivable bacteria

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Abstract Studying the culturable portion of environmental bacterial populations is valuable for understanding the ecology, for discovering taxonomically interesting isolates and for exploiting their enzymatic abilities. In this study, diverse water-related samples, iced water (3 °C) from river, the sediment (29 °C) and water (55 °C) of a hot-spring, were investigated by two cultivation strategies, Dry and novel Wet approach. The isolates were clustered by fluorescent internal transcribed spacer PCR and identified by 16S rRNA sequencing. Several bacterial groups were also sub-typed through the application of Random Amplified Microsatellite Polymorphisms method. A broad enzymatic screening of all bacterial isolates was performed in order to assess the proteolytic, cellulolytic, lipolytic, esterolytic, amylolytic properties, as well as catalase and peroxidase activities. The Wet cultivation demonstrated to be suitable for the isolation of potential new species belonging to genera *Massilia*, *Algoriphagus*, *Rheinheimera* and *Pandoraea*. Valuable microbial resources with extensive enzymatic activities were recognized among the psychrophilic (*Pantoea brenneri* and *Serratia* sp.), mesophilic (*Pandoraea*, *Massilia*, *Pseudomonas*, *Stenotrophomonas*, *Bacillus* and *Aeromonas*) and thermophilic bacteria (*Aeribacullus pallidus* and *Geobacillus kaustophilus*). The experimental strategy developed in this study includes simple investigation tools able to reveal the genetic and enzymatic peculiarities of isolated microorganisms. It can be applied to different kinds of aquatic samples and extreme environments similar to those described in this study.

Keywords Water-related samples · Wet cultivation · f-ITS · RAMP · Hydrolytic abilities · Antioxidant enzyme activities

Introduction

Microbial communities are complex biological structures responsible of different natural processes, which can colonize several kinds of environments. In the "omics" era, the microbiological investigation regards the identification of microorganisms and assessing their properties without cultivation. On one hand these kinds of studies increase the knowledge of environmental bacterial communities and of rare bacterial groups (Albertsen et al. 2013; Kolmakova et al. 2014; Song et al. 2013) but, on the other hand, most microorganisms in the environment remain uncultivated and are basically unexplored. Usually the cultivation of bacteria by traditional agar media permits the isolation of fast growing species at the expense of the slow growing and also rare ones (Prakash et al. 2013). This is caused by the composition of the media utilized, as on media rich in nutrients, the active and fast growing bacteria have a big advantage over the many other bacteria of the same environment, which are in a dormant state. In many natural environments, such as soil and water, the dormant state of bacteria is a survival mechanism employed when nutrients are scarce and conditions adverse (Hoehler and Jørgensen 2013). Discovering these microorganisms and bringing

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them into culture is essential for progress in both basic and applied microbiology.

Hot-springs and iced freshwater can be considered unfavorable habitats where the limited availability of nutrients is coupled to high or low temperatures, respectively. These environments can be considered as microbial reservoirs composed of several interesting groups of bacteria adapted and living in extreme conditions, which are consequently able to produce useful enzymes (Arnosti et al. 2014; Margesin and Miteva 2011; Van Den Burg 2003). Therefore, isolation and screening of the enzymatic abilities of bacteria present in these environments are important issues connected to microbial ecology, but also to taxonomy and biotechnology.

Novel cultivation strategies are necessary in order to provide access to the greatest part of microbial diversity: unexplored species missed until now. Recently, different cultivation approaches were proposed, which put in strict contact the microbial communities with the environment of origin (Bollmann et al. 2007; Nichols et al. 2010; Park et al. 2011).

In this study, a novel cultivation strategy, Wet approach, able to establish a relation between the bacterial assemblage and original water environment, was developed and compared with a Dry cultivation approach, where the connection bacteria—environment was avoided. These two cultivation strategies included two diverse agar media with oligotrophic compositions, which were incubated at different temperatures.

Another crucial peculiarity of culture-dependent analysis of environmental samples is, very often, the selection of the right colonies from an excess of colonies of indistinguishable morphology, in order to obtain a plausible picture of the microflora in the studied environment. In this study, in order to accelerate and simplify the process of selection and clustering of isolated microorganisms, fluorescent-Internal Transcribed Spacer PCR (f-ITS) was performed and coupled to 16S rRNA sequencing for identification of cluster-representative isolates. The isolated bacteria were also genetically characterized by Random Amplified Microsatellite Polymorphisms (RAMP; Chebeňová et al. 2010). Finally, all isolates were easily screened by the use of specific agar media regarding their proteolytic, cellulolytic, lipolytic, esterolytic and amylolytic properties, and by assays that assessed their catalase and peroxidase activities. This multistep procedure permitted effective genetic and enzymatic characterization of isolated bacteria. The developed investigation strategy is suitable for analysis of the cultivable portion of the bacterial microflora of various kinds of water-related environments.

Materials and methods

Sampling, bacterial isolation and growth conditions

Water samples were collected from two different extreme environments. The first set of samples was collected from a hot spring in Gabčíkovo, Slovakia (N 47.8897838391662 E 17.597094923257828, 117 m above sea level). Slovakia is rich in thermal springs; mainly in the area called "Žitný ostrov" where also various spa facilities (Dunajská Streda, Vel'ky Meder and Gabčíkovo) are present. During the nineties of the twentieth century, a drilling of about 460 m was performed in order to use the water for a thermal park. Unfortunately, the park was not constructed and the water is collected, for many years, inside an artificial pond (Fig. 1a). From Gabčíkovo, samples of water at temperature of 55 °C and sediment, which had a temperature of 29 °C, were collected; the samples were recovered from a free-access place which did not require any special permission from spa officers.

Another water sample was recovered from an iced branch of the Danube River (Fig. 1b; Chorvátske rameno, Bratislava, Slovakia; N 48.11568707011708 E 17.106497436761856, 136 m above sea level) at a temperature of 3 °C on February 2014 after about 1 week with a temperature of −7 and −8 °C (Fig. 1c). The sample from the Danube River branch (Chorvátske rameno) was used in order to challenge the multistep procedure with an environment totally different from the hot-spring. Both sites were chosen for their proximity to our laboratory, such factor permitted a rapid and proper treatment of the samples.

Several liters of water samples (from the hot spring and from the Danube) were filtered through different cellulose nitrate hydrophilic membranes (Sartorius Stedim Biotech, Göttingen, Germany) of a 50 mm diameter with a 0.2 µm pore size (2 l of water for each membrane).

Two-hundred-fifty grams of the sediment sample from Gabčíkovo was mixed with 500 ml of Gabčíkovo sterile water and filtered through Whatman 1 filter paper (Merck, Darmstadt, Germany). The obtained sediment extract (500 ml) was then filtered through the cellulose nitrate membrane filter mentioned above. The filtration through the cellulose nitrate membrane was performed in order to concentrate the bacterial microflora of the sediment extract in the membrane.

Then, the membranes containing water samples (Danube and Gabčíkovo hot spring) and those with Gabčíkovo sediment extract were placed on 60 mm agar plate of M9 (own formulation) and tenfold-diluted Luria–Bertani (Sambrook et al. 1989) and each sample was incubated at 3, 28 and 55 °C; this strategy was called Dry Cultivation

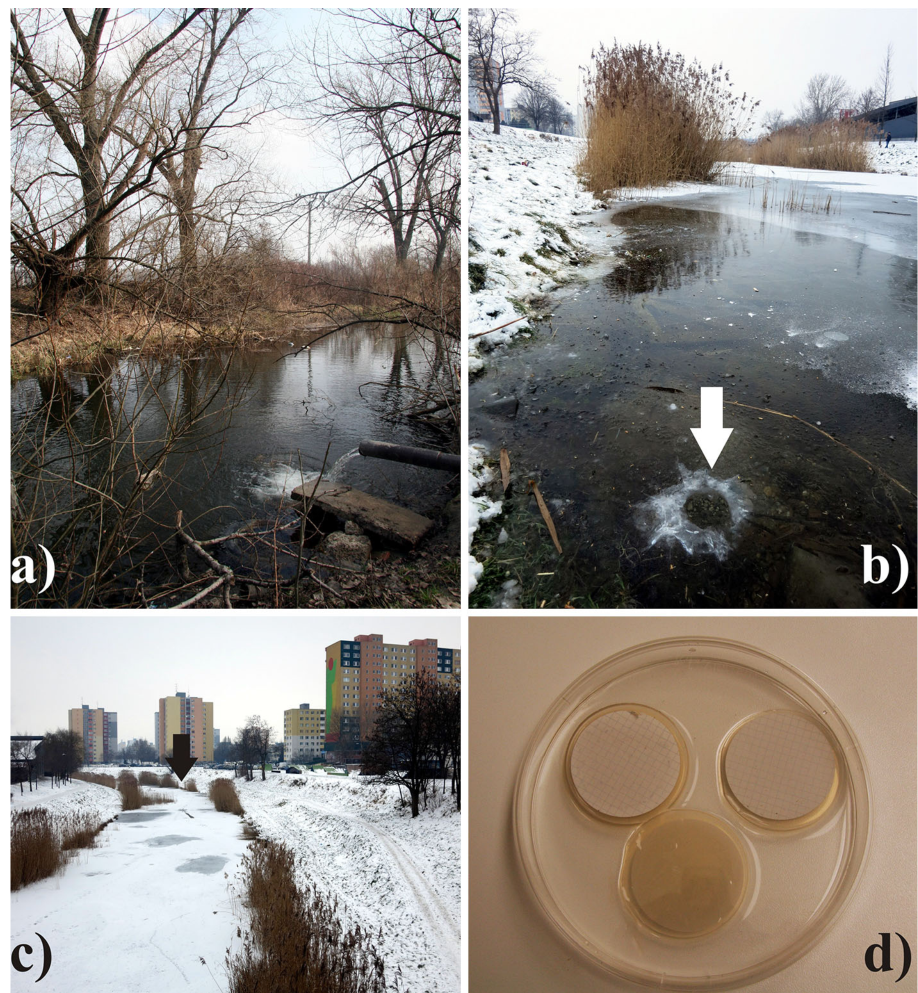


Fig. 1 Sampling environments and the Wet cultivation system. **a** Gabčikovo environment with the water source and the pond. **b** The hole (*white arrow*) in the iced surface of the Danube branch (Chorvátske rameno) from where the water sample was recovered. **c** The urban winter panorama with the iced Danube branch and the sampling place (*black arrow*). **d** Example of the Wet cultivation system including the agar media discs with, on the *top*, the nitrocellulose membrane immersed in the environmental water. (Color figure online)

System. The Dry strategy is very similar to fairly well-known membrane filtration method used for the cultivation of bacteria from water samples (Rompré et al. 2002; Allen et al. 2004).

The medium M9 was composed of the main fraction containing 15.12 g l^{-1} Na_2HPO_4 $12H_2O$, 3.0 g l^{-1} KH_2PO_4, 0.5 g l^{-1} NaCl, 1.0 g l^{-1} NH_4Cl and 15 g l^{-1} agar dissolved in 979 ml of filtered environmental water (from Danube or from hot-spring) or of the sediment extract. The M9 medium was completed adding 100 µl of 1 M $CaCl_2$, 1 ml of 1 M $MgSO_4$ $7H_2O$, 500 µl of 2000 × diluted thiamine (sterilized by filtration) and 20 ml of a 20 % glucose solution (sterilized separately by autoclaving) to the previously autoclaved main fraction. LB10 medium was prepared by dissolving 1 g l^{-1} peptone, 0.5 g l^{-1} yeast extract, 1 g l^{-1} NaCl and 15 g of agar to 1000 ml of filtered environmental water.

Another cultivation strategy (Wet Cultivation System) was performed by gently removing the agar media (M9 and LB10) from 60 mm Petri dishes and placed into the filtered water or filtered sediment suspension of the environment of isolation (from Danube or from the hot spring) inside a Petri dish of 150 mm of diameter. The membranes with trapped bacterial isolates were placed on the agar discs of M9 and LB10, which were in contact with the filtered water or with the filtered sediment suspension (Fig. 1d). Different replicas of this cultivation system containing either the Danube or the hot-spring samples (water and sediment), were produced and each sample was incubated at 3, 28 and 55 °C. Subsequently, the isolates were purified by several spreadings on either M9 or LB10 medium and incubated at the same temperature of as at their first isolation.

DNA extraction, f-ITS clustering and 16S rRNA identification

Bacterial chromosomal DNA was extracted using the InstaGene Matrix (Biorad, Hercules, CA, USA) following the instructions of the manufacturer.

The bacterial isolates were clustered by fluorescent ITS PCR (f-ITS PCR). The internal transcribed spacer between

16S and 23S rRNA genes was amplified, followed by separation of fluorescently labeled PCR products by capillary electrophoresis according to Pangallo et al. (2013). This PCR assay exploits the multiple copies of the ITS region and its length polymorphism inside the bacterial genome among different bacterial strains. Amplification of the ITS region produces species-specific profiles (including different amplicons with different lengths), which permit to cluster the isolated bacteria.

Representative strains of each f-ITS cluster were identified by 16S rRNA sequencing using the primers 27f (5′ agagtttgatcctggctcag 3′) and 685r (5′ tctacgcatttcaccgctac 3′) (Lane 1991). The final PCR mixture (25 µl) consisted of 50 pmol of each primer, 200 µmol l^{-1} of dNTP, 1.5 U DFS Taq DNA polymerase (Bioron, Ludwigshafen, Germany), 1 × PCR buffer, and 6 µl of the template DNA solution. The temperature program consisted of initial denaturation at 95 °C for 1 min, 30 cycles (95 °C for 10 s, 54 °C for 20 s, 72 °C for 1 min) and a final polymerization step at 72 °C for 8 min. This PCR assay was used because Kim et al. (2011) demonstrated how the 16S rRNA amplicon (V1–V4 region), produced by the primer pair 27f and 685r, can be considered reliable almost as full-length sequence of the gene (about 1500 bp). In addition, this small amplicon of about 650 bp can be easily sequenced by one run and rapidly processed.

The PCR products of amplification of the 16S rRNA gene were purified using ExoSAP-IT (Affymetrix, Cleveland, USA) and then sequenced by a commercial facility (GATC Biotech, Constance, Germany). The obtained sequences were compared with those present in the GenBank database using a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences were deposited in GenBank under the accession numbers KP862679–KP862743.

DNA-typing by RAMP

RAMP-PCR was applied in order to characterize the isolates on subspecies level. We applied a protocol similar to our previous study (Chebeňová et al. 2010). Amount of 50–100 ng DNA was added to the reaction mixture (final volume 25 µl) containing 2 U SuperHotTaq DNA Polymerase (Bioron), 1× PCR buffer, 2.4 $mmol^{-1}$ $MgCl_2$, 500 $µmol^{-1}$ dNTP, 60 pmol of the microsatellite primer M13 (5′gagggtggcggttct 3′; Huey and Hall 1989) and 60 pmol of the random primer K7 (5′-caactctctctctct-3′; Wu et al. 1994). PCR program consisted of initial denaturation at 95 °C for 5 min, 30 cycles (95 °C for 45 s; 30 °C for 60 s with ramping 0.1 °C s^{-1} to 50 °C; 50 °C for 60 s with ramping 0.1 °C s^{-1} to 68 °C; 68 °C for 90 s) and a final polymerization at 68 °C for 10 min. Five microliters of RAMP-PCR products were visualised on 1.8 % agarose gel for 4.5 h at 2.3 V cm^{-1} in TAE buffer. Gels were stained with ethidium bromide, visualized under UV light, photographed and digitalized with computer analysis.

Hydrolytic activities

The proteolytic activity of isolated microorganisms was tested by the use of casein agar medium composed of skim milk (250 ml) fractionally sterilized at 100 °C for 30 min (daily within 3 days) and Nutrient agar No 2 (750 ml; Biomark, Pune, India) added, immediately after sterilization, to warm sterilized skim milk. Another proteolytic assay was represented by the hydrolysis of gelatin on agar plate composed of 0.5 g l^{-1} KH_2PO_4, 0.25 g l^{-1} $MgSO_4$, 4 g l^{-1} gelatin and 15 g l^{-1} of agar in distilled H_2O. The medium was sterilized by autoclaving and poured into Petri dishes.

Lipase activity was tested on Spirit blue medium (Himedia, Mumbai, India) following the instructions of the manufacturer. Spirit blue agar, 32.15 g, was suspended in 1000 ml of distilled water and heated by boiling in order to dissolve the medium completely. Medium was sterilized by autoclaving and then cooled to 50 °C. Then, 30 ml of the lipase substrate (1 ml of Tween 80 dissolved in 400 ml warm distilled water, added 100 ml of olive oil, stirred vigorously and autoclaved) was added to the medium.

Esterase activity was detected on Tween 80 agar containing 10 g of peptone, 5 g NaCl, 100 mg $CaCl_2$, 15 g of agar in 1000 ml of distilled H_2O. The medium was adjusted to pH 7.5, autoclaved and cooled to about 60 °C. Then, 10 ml of separately autoclaved Tween 80 was added. Esterase producing microorganisms showed a precipitation of calcium salt and fatty acids around their colonies.

Celullolytic ability was checked on Congo Red agar plates; this medium contained 0.5 g l^{-1} KH_2PO_4, 0.25 g l^{-1} $MgSO_4$, 2 g l^{-1}cellulose, 0.2 g l^{-1} Congo red, 2 g l^{-1} gelatin, and 15 g l^{-1} agar in distilled H_2O. The pH of the medium was adjusted to 6.8-7.2 and it was autoclaved.

The amylolytic property were assayed on agar medium containing 10 g l^{-1} soluble starch, 5 g l^{-1} peptone, 5 g l^{-1} yeast extract, 0.5 g l^{-1} $MgSO_4$ 7 H_2O, 0.01 g l^{-1} $FeSO_4$ 7 H_2O, 0.01 g l^{-1} NaCl, 15 g l^{-1} agar and distilled H_2O.

Each microorganism was plated into the center of different 60 mm Petri dishes containing the one of the specific media described above. All the agar assays were performed in triplicate and the positive reaction, except of that for Tween 80 agar, was displayed as a zone of clearance (hydrolysis) around the assayed bacterial colonies.

Catalase and peroxidase activities

In order to assess the catalase and peroxidase activities, bacterial cultures were grown in 5 ml of Luria–Bertani

medium (10 g tryptone, 5 g yeast extract and 5 g NaCl in 1000 ml) at 3, 28 or 55 °C depending on the type of isolates. Grown cultures were transferred (0.5 ml) to 50 ml of Luria–Bertani medium and incubated at 3, 28 or 55 °C under shaking at 200 rpm. When growth reached the middle of the exponential phase (optical density 0.5 at 610 nm), the cells were harvested by centrifugation at 6000 rpm for 10 min at 4 °C and washed with phosphate-buffered saline solution (PBS buffer; 8.0 g l^{-1} NaCl; 0.2 g l^{-1} KCl; 1.44 g l^{-1} Na_2HPO_4; 0.24 g l^{-1} KH_2HPO_4; pH 7.0) and frozen. The mechanical disruption of frozen cells was performed in 50 mM sodium phosphate, 2 mM EDTA (pH 7.5) at 4 °C with glass beads (diameter 0.4 µm) using the Disruptor Genie (Scientific Industries, New York, USA) for 5 min. The homogenates were centrifuged at 14,000 rpm for 10 min. Supernatants were used for the determination of enzymatic activities. Catalase (CAT; EC 1.11.1.6) activity was determined at pH 7.0 by monitoring the decomposition of H_2O_2 at 240 nm with an extinction coefficient of 43.6 M^{-1} cm^{-1}. One unit of catalase activity (U) was defined as the amount of enzyme that catalyzes the decomposition of 1 µmol of H_2O_2 per minute (Roggenkamp et al. 1974).

Peroxidase (POX; E.C.1.11.1.7) activity was assayed at pH 5.5 by monitoring the oxidation of *o*-dianisidine dihydrochloride at 460 nm with an extinction coefficient of 11.4×10^3 M^{-1} cm^{-1}. One unit of peroxide activity (U) was defined as the activity that produces 1 µmol of oxidized *o*-dianisidine per minute (Claiborne and Fridovich 1979). The assays to assess the CAT and POX activities were performed in triplicate.

Results

Cultivation strategies

When Dry and Wet strategies were compared, the samples from Gabčikovo hot spring (water and sediment) showed some differences regarding their bacterial diversity (Fig. 2a, b), while the two microbiological media (M9 and LB10) were able to recover almost the same kind of species. From water samples, a total of 19 bacteria (9 by Dry and 10 by Wet strategy) were isolated, belonging mainly to the Bacillales family (*Aeribacillus*, *Bacillus* and *Geobacillus*). Only two isolates (*Massilia*) belonged to order Burkholderiales (Fig. 2a; Table 1S).

The Dry strategy was more effective in recovering different bacterial members (28 isolates) from hot-spring sediment compared to Wet strategy (17 isolates). The most frequently isolated bacteria, by Dry strategy, belonged to the genus *Aeribacillus* (10 isolates), while the common characteristic of the two strategies regarded the isolation of the same numbers of *Aeromonas* isolates (7 isolates for both strategies). The Wet strategy had the ability to recover a larger number of *Rheinheimera* isolates (3) and one *Algoriphagus* member, compared to the Dry approach. Members of the genera *Aeribacillus* and *Geobacillus* were the only isolates recovered from sediment after the incubation at 55 °C (Fig. 2b). From hot-spring samples, none microorganism was isolated by the incubation at 3 °C.

A different bacterial microflora was recovered from iced water; various psychrophilic bacteria were isolated only by the Wet strategy after about 18 days of incubation at 3 °C. These strains belonged to the genera *Pantoea*, *Pseudomonas*, *Rahnella* and *Serratia* (Fig. 3a). All these strains, except of two *Pseudomonas*, were recovered on M9 medium; although on LB10 more bacteria (16) were isolated respect to M9 (13), using both Dry and Wet approaches. Members of the genus *Bacillus* were recovered by both strategies and media.

By Wet strategy, a bigger bacterial diversity was recovered compared to Dry approach. On Dry plates, only mesophiles were able to grow (at 28 °C) and just one thermophilic bacterium belonging to the genus *Geobacillus*, which was also isolated on LB10 medium in Wet condition (Fig. 3a, b).

Clustering, identification and genotyping

Ninety-three bacteria were isolated from different water-related samples; 19 and 45 isolates from hot-spring water and sediment, respectively; and other 29 isolates from iced water of the Danube River branch. The f-ITS permitted to group the isolated bacteria, several species-specific profiles (45) being produced. In this way, one or just a limited number of bacterial representatives of each f-ITS group were identified by 16S rRNA sequencing.

Bacillus members formed the biggest mesophilic group; it contained 21 isolates, which were divided to 10 different f-ITS clusters. Several *Bacillus* isolates composed alone an f-ITS group. The isolates identified as *Bacillus amyloliquefaciens* and *B. safensis* were grouped in two separate assemblages with their typical f-ITS profiles (Table 1).

The isolates belonging to *B. aryabhattai* and *B. firmus* groups exhibited two diverse f-ITS profiles for each group. The genetic variability of these two species, especially *B. aryabhattai*, was also evidenced by RAMP PCR. Indeed, the 5 *B. aryabhattai* isolates were genotypically divided in three RAMP groups (Bc-A–Bc-C; Table 1). The *B. aryabhattai* were the only bacteria isolated both from hot-spring sediment and iced water.

The second biggest mesophilic group was represented by the *Aeromonas* assemblage. Three *Aeromonas* species were identified (*A. hydrophila*, *A. media* and *A. punctata*). The *A. hydrophila* and *A. media* isolates demonstrated to

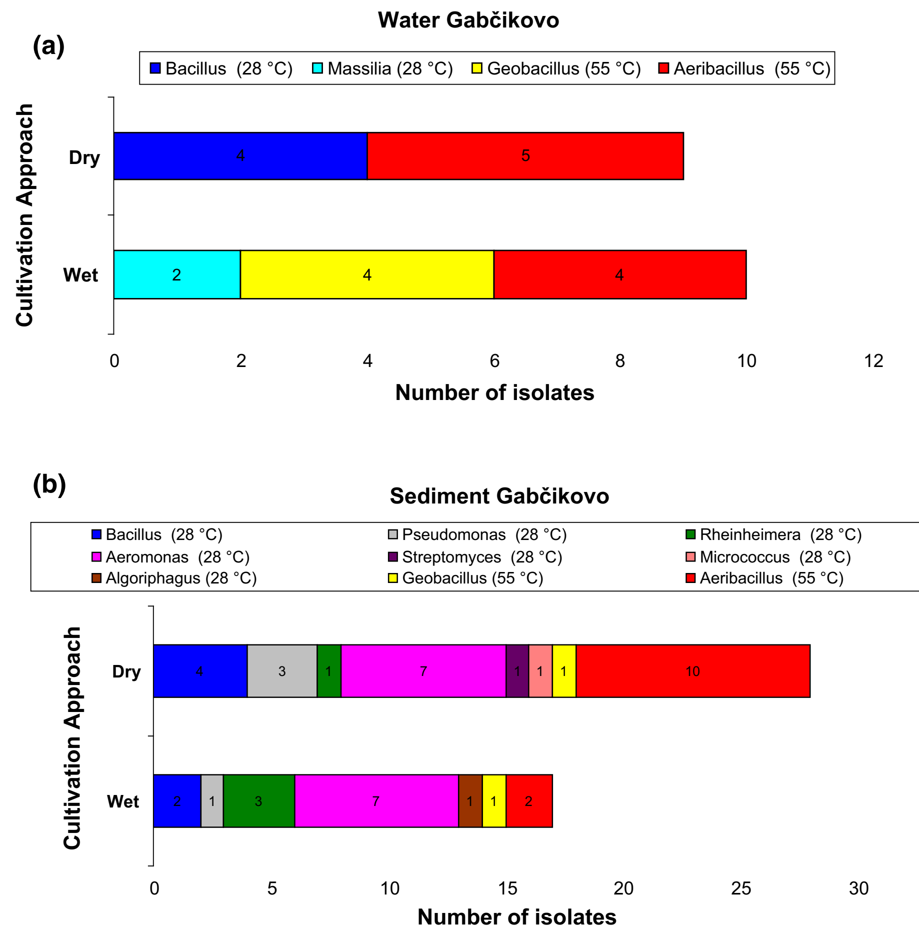


Fig. 2 Comparison of bacterial isolates recovered from Gabčikovo hot-spring samples (water and sediment) using two different cultivation strategies, Dry and Wet

have intra-species variability and they were divided into 3 and 2 different f-ITS profile, respectively. Such genetic variability was confirmed also by RAMP PCR, which located seven *A. hydrophila* isolates in 5 different groups; and the six *A. media* isolates produced 4 diverse RAMP profiles (Table 1).

The four *Rheinheimera* isolates produced 3 f-ITS profiles, but each isolate was characterized by its-own RAMP fingerprint. The genetic divergence of the four *Pandoraea* isolates was displayed by two different f-ITS profiles and three typical RAMP genotypes (Table 1).

The thermophilic bacteria appeared to be a more homogenous group. Three *Geobacillus thermoglucosidasius* produced only one f-ITS and one RAMP profile. Two *G. thermodenitrificans* isolates presented two typical f-ITS profiles, but they produced only one RAMP fingerprint. Three over four isolates of *G. kaustophilus* were included in the same f-ITS and RAMP clusters. Twenty-one isolates belonging to the species *Aeribacillus pallidus* were characterized by the same f-ITS profile, but they were divided in two different groups by RAMP PCR; the profile Ar-A included 2 members, while 19 isolates fitted in the group Ar-B (Table 1).

Hydrolytic properties and antioxidant enzyme activities

Only 5 isolates, *Geobacillus thermodenitrificans* M9-G-73-55-W-W and four *Aeribacillus pallidus* (LB10-G-14-55-S-D, LB10-G-67-55-S-D, M9-G-27-55-W-W and M9-G-74-55-W-W), did not produce hydrolysis on any plate assay. Figure 4 and Table 1S show the hydrolytic and CAT/POX activities of the isolated bacteria.

The isolates from hot-spring sediment had significant proteolytic and lipolytic properties, mainly the bacteria isolated by Wet cultivation at 28 °C (Fig. 4a). The most active bacteria belonged to the genera *Bacillus* and *Aeromonas* (Table 1S); in addition to hydrolytic abilities, different isolates evidenced high CAT activity such as *B. firmus* M9-G-7-28-S-D (400 U/ml) and *A. media* M9-G-18-28-S-D (400 U/ml) or M9-G-21-28-S-D *Bacillus aryabhattai* (370 U/ml) and *A. hydrophila* M9-G-54-28-S-D (450 U/ml).

Among the isolates grown at 55 °C, *Geobacillus kaustophilus* LB10-G-69-55-S-D was positive in proteolytic, lipolytic, cellulolytic and amylolytic assays and expressed a high POX activity (0.3 U/ml); while four *Aeribacillus*

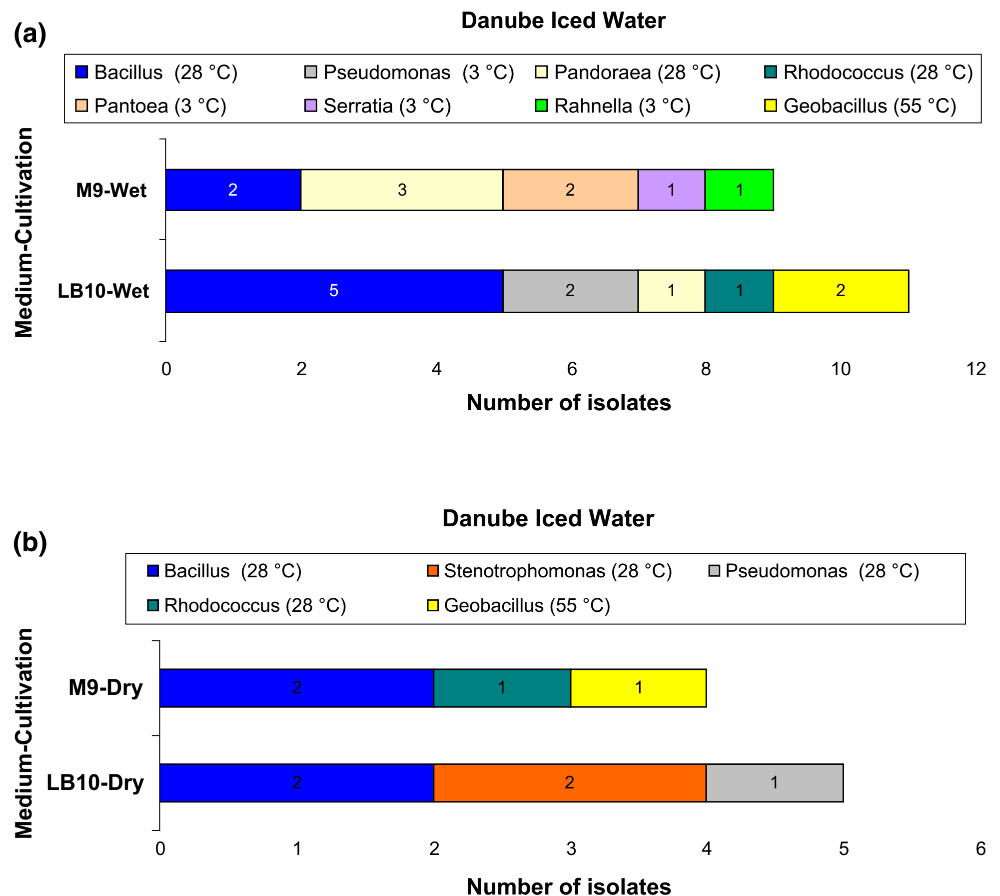


Fig. 3 Comparison of bacterial isolates recovered from Danube iced water using two different cultivation strategies, Dry and Wet

pallidus exhibited catalase activity with values from 450 to 510 U/ml.

The water sample from hot spring was largely characterized by thermophilic bacteria which displayed weaker enzymatic activities compared to bacteria isolated from sediment (Fig. 4b). *Aeribacillus pallidus* isolates displayed high CAT activity, for the strain M9-G-72-55-W-D a value of 800 U/ml being determined. On the other hand, two *Geobacillus kaustophilus* showed a significant POX activity of 0.3 U/ml, and the strain LB10-G-30-55-W-W together with two *Massilia* isolates were able to hydrolyze proteins, lipids, cellulose and starch. Four mesophilic *B. firmus* isolates evidenced weak hydrolytic properties (Table 1S).

The psychrophilic isolates from Danube iced water displayed a widespread lipolytic activity (83 % of isolates). Such characteristic was also expressed by mesophilic isolates, indeed 100 % of bacteria cultivated by Wet approach were positive in the Spirit Blue test. Other widespread activities were casein and cellulose degradation (87 % of bacteria isolated by Dry cultivation), starch hydrolysis (83 % of isolates by Wet approach) and catalase activity (100 % of isolates; Fig. 4c).

The members belonging to *Bacillus* genus from Danube iced water were generally the most active group. *B. amyloliquefaciens* isolates produced positive reactions in all plate assays. Other mesophilic bacteria such as *Stenotrophomonas* sp. LB10-CHR-13-28-D and *Pandoraea pnomenusa* M9-CHR-12-28-W evidenced interesting proteolytic, lipolytic, cellulolytic and amylolytic abilities. Isolates of this sample demonstrated to have a significant CAT activity; several strains such as *Pantoea agglomerans* M9-CHR-1-3-W, *Pantoea brenneri* M9-CHR-6-4-W, *Bacillus safensis* M9-CHR-4-28-D and LB10-CHR-1-28-W achieved values 800 U/ml and higher, and *Pandoraea* sp. LB10-CHR-10-28-W even 1402 U/ml. In addition, bacteria isolated at 4 °C possessed also relevant POX abilities (from 0.3 to 0.5 U/ml) and the strain *Serratia* sp. M9-CHR-4-3-W was able to hydrolyze quickly and extensively casein, Tween 80 and cellulose at 3 °C (Table 1S).

Discussion

In this study, we tried to develop a new cultivation strategy in order to recover not yet cultivated bacteria from water-related samples considered as extreme environments. Such strategy was inspired by the diffusion chamber proposed by

Table 1 f-ITS clustering, RAMP genotyping and phylogenetic classification of representative isolates

Bacterial isolates	f-ITS profile	RAMP cluster	Closest phylogenetic relative
LB10-CHR-3-28-W	282, 474	Bc-A	KM659228 *Bacillus aryabhattai*/KJ526881 *Bacillus megaterium* 100 % (633/633)
LB10-CHR-8-28-D; LB10-CHR-11-28-D; M9-G-35-28-S-D	303, 412	Bc-B	
M9-G-21-28-S-D	303, 412	Bc-C	
M9-G-7-28-S-D	220, 370, 390, 410	Bc-D	LC019792 *Bacillus firmus*/KM873030 *Bacillus oceanisediminis* 100 % (617/617)
LB10-G-63-28-W-D; LB10-G-56-28-W-D; M9-G-48-28-W-W; M9-G-49-28-W-W	376, 495, 598	Bc-E	
LB10-CHR-1-28-W	260, 387, 413	Bc-F	KM603646 *Bacillus safensis*/KJ094439 *Bacillus pumilus* 100 % (616/616)
M9-CHR-4-28-D			
LB10-CHR-2-28-W; M9-CHR-6-28-D; M9-CHR-7-28-W; LB10-CHR-9-28-W; M9-CHR-10-28-W	260, 315, 385, 415	Bc-G	KP036929 *Bacillus amyloliquefaciens*/KJ944089 *Bacillus* sp./KF217257 *B. methylotrophicus* 100 % (633/633)
			KJ538551 *Bacillus amyloliquefaciens* 100 % (633/633)
LB10-CHR-5-28-W	258, 385, 422	Bc-H	KM823958 *Bacillus subtilis*/KM437882 *Bacillus tequilensis* 100 % (627/627)
LB10-G-60-28-S-W	253, 387, 415	Bc-I	KP065495 *Bacillus* sp. 100 % (615/615)
LB10-G-32-28-S-W	315, 440, 560	Bc-J	KF740857 *Bacillus* sp. 100 % (593/593)
M9-G-17-28-S-D	237, 402	Bc-K	KP119811 *Bacillus licheniformis* 100 % (553/553)
LB10-G-62-28-S-W	521	Rh-A	DQ914845 Gamma proteobacterium 100 % (600/600)/ GQ464399 Uncultured *Rheinheimera* sp. 99 % (599/600)
LB10-G-11-28-S-W	496	Rh-B	FM201201 Uncultured bacterium 99 % (604/606)/ GQ284452 *Rheinheimera taxanensis* 99 % (602/606)
LB10-G-58-28-S-D	521	Rh-C	HQ433560 Uncultured bacterium clone 100 % (599/599)/ GQ464399 Uncultured *Rheinheimera* sp. 99 % (598/599)
LB10-G-34-28-S-W	515, 596, 628, 690, 733	Rh-D	JN868743 Uncultured bacterium 100 % (587/587)/ JX899630 *Rheinheimera* sp. 99 % (586/587)
LB10-G-38-28-S-D; LB10-G-44-28-S-D	540, 569, 608, 675	Ar-A	HF937003 *Aeromonas hydrophila* 100 % (595/595)
LB10-G-10-28-S-D; M9-G-53-28-S-D		Ar-B	
M9-G-20-28-S-W	379, 570, 602, 657	Ar-C	KJ152584 *Aeromonas hydrophila* 100 % (595/595)
LB10-G-57-28-S-W		Ar-D	
LB10-G-46-28-S-W; LB10-G-33-28-S-W; LB10-G-43-28-S-W	548, 585	Ar-E	LN624813 *Aeromonas media* 100 % (602/602)
LB10-G-41-28-S-W		Ar-F	
M9-G-19-28-S-W	570, 588, 672	Ar-G	
M9-G-18-28-S-D		Ar-H	
LB10-G-61-28-S-D	519, 562	Ar-I	GU205198 *Aeromonas punctata* 99 % (626/635)
M9-G-36-28-S-D	590	Ar-J	CP006579 *Aeromonas hydrophila* 98 % (578/591)
LB10-CHR-10-28-W	303, 395, 450, 520, 604	Pd-A	EF080879 *Pandoraea* sp. 95 % (538/567)
M9-CHR-12-28-W	303, 389, 502, 580, 634, 660, 752	Pd-B	AB642180 *Pandoraea pnomenusa* 100 % (628/628)
M9-CHR-13-28-W; M9-CHR-14-28-W		Pd-C	
M9-G-8-28-S-D	561	Ps-A	KF791346 *Pseudomonas* sp. 100 % (605/605)
LB10-G-40-28-S-W	533, 561	Ps-B	KJ855303 *Pseudomonas* sp. 94 % (567/605)
LB10-G-42-28-S-D	220, 578	Ps-C	KM219999 *Pseudomonas alcaligenes* 100 % (617/617)
LB10-G-59-28-S-D	208, 550	Ps-D	DQ859923 *Pseudomonas* sp. 100 % (597/597)
LB10-CHR-2-3-W	587	Ps-E	KM102495 *Pseudomonas* sp./EU723817 *Pseudomonas argentinensis* 100 % (689/689)

Table 1 continued

Bacterial isolates	f-ITS profile	RAMP cluster	Closest phylogenetic relative
LB10-CHR-3-3-W	587	Ps-F	KJ751509 *Pseudomonas* sp. 100 % (646/646)
LB10-CHR-14-28-D	548	Ps-G	KF640247 *Pseudomonas putida* 100 % 639/639
LB10-CHR-2-55-W; LB10-CHR-4-55-W; M9-CHR-5-55-D	320, 403, 511, 578	Gb-A	KJ722454 *Geobacillus thermoglucosidasius* 100 % (636/636)
M9-G-73-55-W-W	357, 410, 463, 533, 630	Gb-B	JX673950 *Geobacillus thermodenitrificans* 100 % (612/612)
M9-G-75-55-S-W	215, 325, 366, 403, 443, 505, 620		FJ823098 *Geobacillus thermodenitrificans* 100 % (637/637)
LB10-G-12-55-W-W; LB10-G-69-55-S-D; LB10-G-30-55-W-W	475, 505,511, 609, 683, 790	Gb-C	KC252984 *Geobacillus kaustophilus* 100 % (634/634)
LB10-G-31-55-W-W	470, 560, 609, 620, 731, 830	Gb-D	
LB10-G-24-55-S-D; LB10-G-70-55-S-D	222, 415, 580	Ab-A	KM596788 *Aeribacillus pallidus* 100 % (632/632)/ KJ842637 *Aeribacillus pallidus* 99 % (621/623)
LB10-G-64-55-W-W; LB10-G-67-55-S-D; LB10-G-68-55-S-D; LB10-G-14-55-S-D; LB10-G-13-55-S-D; LB10-G-25-55-S-D; LB10-G-71-55-S-D; M9-G-15-55-W-D; M9-G-16-55-W-D; M9-G-72-55-W-D; M9-G-22-55-S-W; M9-G-26-55-S-W; M9-G-74-55-W-W; M9-G-27-55-W-W; M9-G-77-55-S-D; M9-G-76-55-S-D; M9-G-29-55-W-D; M9-G-23-55-W-W; M9-G-28-55-W-D		Ab-B	

A code was given to each isolate; for example M9-G-45-28-S-D, where M9 indicates the medium of isolation, G the place of isolation (Gabčikovo), 45 is the serial number of the isolate, 28 the temperature of isolation, S means from sediment (W from water) and D means Dry cultivation strategy. LB10-CHR-5-28-W, LB10—medium, CHR—Chorvátske rameno (Danube), 5—serial number of the isolate, 28—temperature, (from Danube we had only water sample, it was not necessary to divided the isolates with S—sediment or W—water) W—wet cultivation strategy

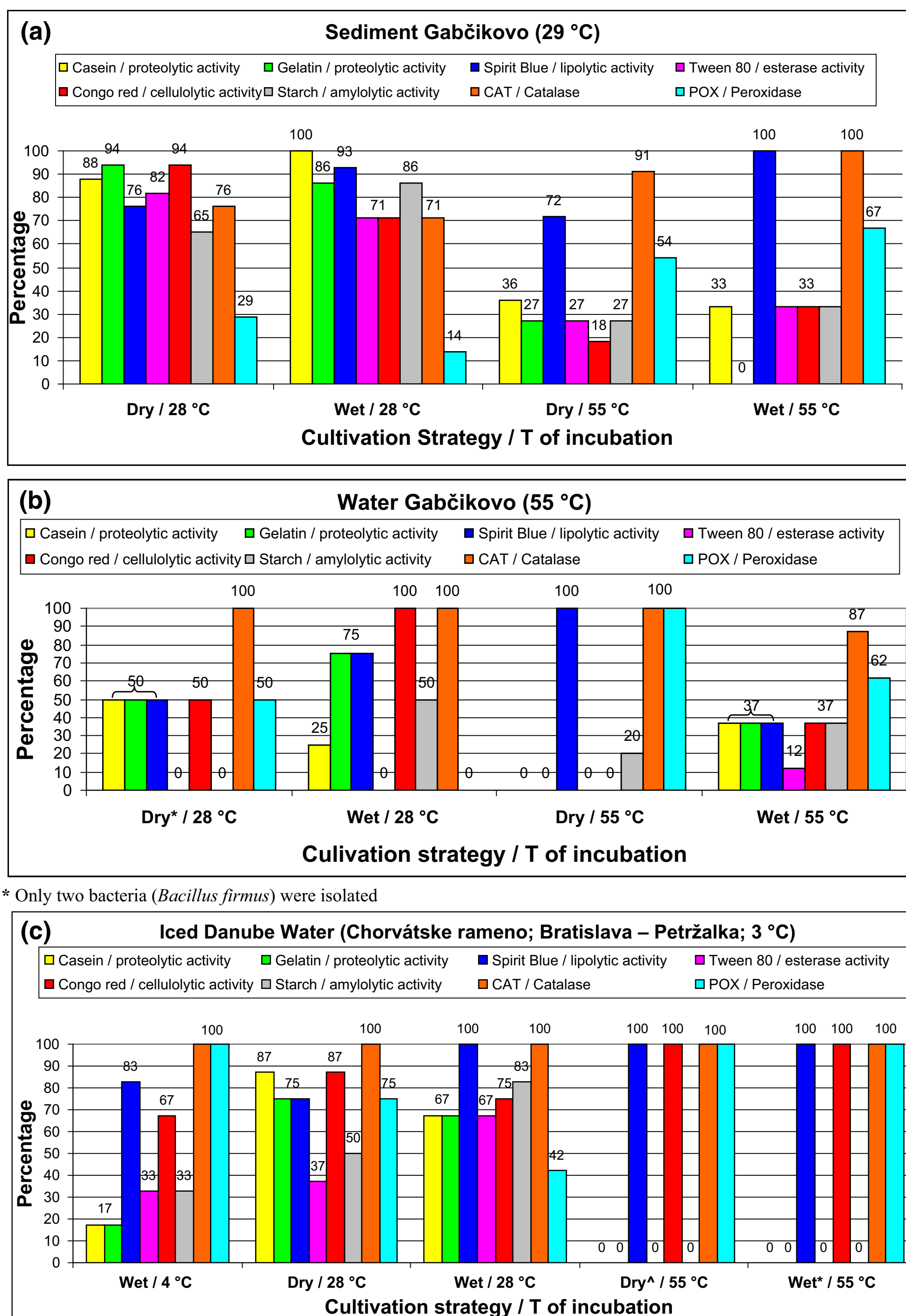
Bollmann et al. (2007). Our cultivation system was simpler to perform, the agar plates with trapped microorganisms inside nitrocellulose filters on the top being directly in contact with filtered water samples (from hot-spring and iced water) or with filtered sediment suspension (from hot-spring) (Fig. 1d). The system, by the use of two media (LB10 and M9) with oligotrophic composition, was able to avoid the bacterial over-growth; it permitted the growth of a suitable amount of isolates, which were easily purified during the subsequent isolation steps.

On the other hand, it is evident that if only one kind of cultivation strategy, Dry or Wet, was employed, only limited results would be achieved. Potentially new species isolated from hot-spring samples included *Massilia* sp., *Algoriphagus* sp., *Streptomyces* sp., several *Rheinheimera*, *Bacillus* and *Pseudomonas* isolates. As the most taxonomically interesting strains, the isolates M9-G-50-28-W-W *Massilia* sp. (GenBank accession number KP862724), LB10-G-9-28-S-W *Algoriphagus* sp. (KP862726) and LB10-G-40-28-S-W *Pseudomonas* sp. (KP862733) could be considered; they evidenced an average 16S rRNA similarity of 96 %. Members of the genus *Pseudomonas* are very well known ubiquitous bacteria with significant adaptation abilities (Silby et al. 2011). On the other hand, the members belonging to genera *Massilia* and *Algoriphagus* are included in relatively new genera described for the first time by La Scola et al. (1998) and Bowman et al. (2003), respectively. For the isolation of *Massilia* members, it was important to incubate some plates of hot-spring water sample in mesophilic condition at 28 °C.

Another interesting group was represented by four *Rheinheimera* strains [LB10-G-62-28-S-W (KP862740), LB10-G-11-28-S-W (KP862741), LB10-G-58-28-S-D (KP862729), LB10-G-34-28-S-W (KP862736)]. The phylogenetic relatedness of their 16S rRNA sequences displayed a 100 % similarity with uncultured bacteria. Moreover, they exhibited variable genotypes by RAMP PCR. *Rheinheimera* genus is also relatively new (Brettar et al. 2002). Members of this genus are isolated and detected in water and sediment samples (Szabó et al. 2011). All these potential new species, except of *Rheinheimera* sp. LB10-G-58-28-S-D, were recovered through the Wet cultivation strategy.

From iced water sample, only few strains such as *Serratia* sp. M9-CHR-4-3-W, *Pseudomonas* sp. LB10-CHR-3-3-W, *Stenotrophomonas* sp. LB10-CHR-12-28-D and LB10-CHR-13-28-D, and *Pandoraea* sp. LB10-CHR-10-28-W can be considered taxonomically interesting. From

Fig. 4 Distribution of enzymatic activities of bacterial isolates from different environments



^ Only one bacterium (*Geobacillus thermoglucosidasius*) was isolated.* Only two bacteria (*G. thermoglucosidasius*) were isolated

this point of view, only the *Pandoraea* sp. strain, which showed a similarity of 95 %, has a high chance to be considered as a new species. *Pandoraea* species are frequently isolated from contaminated aquatic and soil habitats and have interesting biodegradation properties (Chen et al. 2009; Liz et al. 2009).

In our multistep procedure, f-ITS method demonstrated a good capacity to classify the isolated bacteria. Such technique permitted easily to cluster and consequently to identify by sequencing a number of representative isolates. This technique is very useful when many bacteria are isolated and many of them appear very similar; it was

successfully utilized for the classification of different kinds of bacteria recovered from diverse samples (Bučková et al. 2013; Pinar et al. 2014; Pangallo et al. 2015).

RAMP PCR is more suitable to deeply subtype the bacteria belonging to the same species. This feature was clear for various species such as the *Bacillus aryabhattai* group (5 isolates subtyped into three RAMP profiles, Bc-A–Bc-C), *Aeromonas hydrophila* (7 isolates with 5 RAMP profiles), *Aeromonas media* (6 isolates and 4 RAMP profiles), *Pandoraea pnomenusa* (3 isolates divided to two profiles) and *Aeribacillus pallidus* (21 isolates characterized by two different RAMP clusters).

In few cases, the f-ITS classification coincided with the RAMP genotyping, as for example the *Bacillus firmus* and *Geobacillus kaustophilus* groups (Table 1); only in one circumstance the f-ITS had a better discrimination power than RAMP method regarding the subtyping of the *Geobacillus thermodenitrificans* isolates.

The combination of data produced by f-ITS and RAMP PCR, on one hand, evidenced the big variability of some bacterial members, mainly those belonging to genera *Pseudomonas, Rheinheimera, Aeromonas* and *Bacillus*, confirming the results of some previous studies (Maiti et al. 2009; Katara et al. 2012; Vaz-Moreira et al. 2012). On the other hand, it demonstrated the genetic homogeneity of other species such as *Bacillus amyloliquefaciens* and *Geobacillus thermoglucosidasius* (both isolated from iced water) and several *Aeribacillus pallidus* isolates recovered from both hot-spring water and sediment.

It seems that no apparent connection exists between specific genetic fingerprints and enzymatic features. Only in few instances this link was noted, *Bacillus firmus* M9-G-7-28-S-D (f-ITS profile: 220, 370, 390, 410 bp; RAMP profile: Bc-D), *Pandoraea pnomenusa* M9-CHR-12-28-W (RAMP profile Pd-B) and the three *Geobacillus kaustophilus* isolates characterized by the RAMP profile Gb-C (Table 1S), but we can consider it as a simple coincidence.

An important aim of our study was also to recognize the hydrolytic properties and the catalase—peroxidase activities of the isolated bacteria. Such kind of broad screening, in these two different aquatic environments, has been little investigated in previous works, which mainly focused on characterization of a specific group of bacteria (Gugliandolo et al. 2012) or on deep description of the enzymatic abilities of a particular strain (Chen et al. 2011; Ezeji et al. 2005). The strategy to cultivate the microorganisms at three different temperatures permitted the isolation of bacteria producing psychrophilic, mesophilic and thermophilic extracellular enzymes. Among the mesophilic bacteria, *Aeromonas* and *Bacillus* isolates were the most skilled, indeed some *Bacillus* and many *Aeromonas media*, isolated from hot-spring sediment, demonstrated to be able to hydrolyze all the substrates assayed. More active Bacilli were isolated from iced water where the members of the *B. amyloliquefaciens* group evidenced valuable enzymatic features. The enzymatic abilities of *Bacillus* strains were previously largely studied (Schallmey et al. 2004), but data regarding the hydrolytic properties of *Aeromonas* strains isolated from aquatic environments are sporadic, such genus is mainly investigated for its pathogenic characteristics (Janda and Abbott 2010).

Massilia isolates were the most degradative mesophilic bacteria recovered from hot-spring water; they displayed a significant amylolytic ability. These bacteria are ubiquitous (Kämpfer et al. 2011) and their presence in hot and arid environments was investigated only by the culture-independent approach (Nagy et al. 2005; Moquin et al. 2012).

The enzymatic screening allowed to reveal the bacteria producing stable enzymes at extreme temperatures. Psychrophilic bacteria expressed useful enzymes at low temperatures (3 °C), for example the cellulases of *Pantoea brenneri*, *Pseudomonas* sp. and *Serratia* sp. and also the proteases and lipases of the last one. On the other hand, the thermophilic enzymes were produced by the members of *Geobacillus* genus and *Aeribacillus pallidus*. The hydrolytic properties of *Geobacillus* group were already described by different studies (Zeigler 2014). Genus *Aeribacillus* is less characterized in the literature and our study increased the information related to these thermophilic microorganisms.

Physicochemical conditions could influence the growth of bacteria and also their hydrolytic properties. We hypothesized that the low production of hydrolytic enzymes of the isolates from hot-spring water (Fig. 4b; Table 1S) is due to the low concentration of calcium and magnesium ions (Table 2S), which can be considered as co-factors of enzymes.

The knowledge regarding the catalatic and peroxidatic activities can be considered as a novelty for this kind of studies. The catalase and peroxidase enzymes help the bacterial cells against oxidative stress by removing the hydrogen peroxide produced as a byproduct of oxygen metabolism; they can lead to various industrial-technical applications (Xu 2005). So, it is useful to screen the bacterial isolates for such kinds of abilities. To our knowledge, few studies evidenced the presence of catalases and peroxidases in bacteria recovered from water-related samples (Arya et al. 2015). Our investigation showed high catalatic activities in different bacterial isolates: mesophilic isolates belonging to the genera *Aeromonas*, *Pseudomonas*, *Bacillus*, *Pandoraea* and thermophilic *Aeribacillus*. Almost all psychrophilic bacteria evidenced also a significant peroxidase activity.

In conclusion, by this investigation different bacterial isolates from water-related samples subjected to extreme temperature were isolated. Two different strategies were

used (Dry and Wet). Our newly developed Wet strategy seemed to be more suitable for the isolation of taxonomically interesting bacteria, although the combination of the two strategies and media allowed the isolation of different kinds of bacterial species. This study again emphasized the need for new cultivation approaches to increase the knowledge regarding environmental bacterial communities.

The f-ITS clustering was able to group the isolates and to select the representative strains in order to be identified by sequencing. The RAMP genotyping showed, mostly, the relations among the isolates belonging to the same species isolated by different strategies or media.

The hydrolytic and antioxidant enzymatic screening evidenced the important characteristics of isolates to be utilized in several kinds of applications. The agar tests and the biochemical assays showed, in an easy way, the enzymatic characteristics of the isolates and facilitated consequent selection and collection of the most promising bacteria. The potential of thermophilic and psychrophilic enzymes was discussed in many previous studies, which underlined their valuable contributions in diverse industrial and biotechnological processes (Xu 2005; Cavicchioli et al. 2011; Bergquist et al. 2014).

Our multistep procedure was applied to different water environments in order to prove its effectiveness. We suggest that it is used for studying other kinds of aquatic samples and using other types of microbiological media in order to isolate and select valuable bacterial resources.

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