Extremophiles (2008) 12:763–773
DOI 10.1007/s00792-008-0183-5

ORIGINAL PAPER

Phylogenetic and metabolic bacterial diversity of *Phragmites australis* periphyton communities in two Hungarian soda ponds

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Received: 19 March 2008 / Accepted: 7 July 2008 / Published online: 5 August 2008

Abstract Bacterial diversity of reed (*Phragmites australis*) periphyton communities of Kelemen-szék and Nagy-Vadas (two Hungarian soda ponds) was investigated using molecular cloning and cultivation-based techniques. The majority of the 80 Kelemen-szék and 72 Nagy-Vadas bacterial isolates proved to be moderately halophilic and alkaliphilic. A great proportion of the isolates showed phosphatase and urease activity, utilized aesculin, citrate and certain biopolymers (e.g., gelatine and tween 80). Partial 16S rDNA sequence analysis of 33 Kelemen-szék and 20 Nagy-Vadas ARDRA group representatives showed Gram-positive (*Nesterenkonia*, *Cellulomonas*, *Dietzia*, *Bacillus* and *Planococcus*) dominance at both sampling sites. Species of the genera *Acidovorax*, *Hydrogenophaga* (β-Proteobacteria) and *Flavobacterium*, *Sphingobacterium* (Bacteroidetes) were represented only from Kelemen-szék. Altogether 16 isolates showed low sequence similarity with yet described bacteria and may represent novel taxa. Screening of the 16S rRNA gene libraries of 129 Kelemen-szék and 158 Nagy-Vadas clones resulted in 30 and 28 different ARDRA groups, respectively. Sequence analysis revealed a Gram-negative (*Rheinheimera*, *Aquimonas*, *Cellvibrio*, *Flavobacterium* and *Sphingobacterium*) dominated phylogenetic diversity. A high number of the clones were affiliated with uncultured bacterial clones described from diverse environmental samples.

Communicated by T. Matsunaga.

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Keywords *Phragmites australis* · Biofilm · Cultivation · Cloning · 16S rRNA sequence analysis

Introduction

Saline and alkaline aquatic environments are natural resources of great importance all over the world due to their unique geological, hydrological, botanical, zoological and microbiological characteristics. Comparing to soda lakes situated elsewhere, the soda lakes of the Carpathian-basin (Hungary) can be characterized by relatively low salt-content and high pH values. The average salt concentration fluctuates between 0.5 and 7.5 g l^{-1}, but in the case of certain water bodies this value can reach 70 g l^{-1} during summer because of the high evaporation rate. The water bodies of Kiskunság National Park (e.g., Kelemen-szék) are characterized with higher salt-content. Conductivity values vary between 1,600 and 10,500 μS cm^{-1} whereas pH fluctuates between 8.1 and 10. The soda ponds of Hortobágy National park (e.g., Nagy-Vadas) show similar pH values but lower values of conductivity, up to 6,000 μS cm^{-1} (Boros 1999).

The dominant emergent macrophyte in soda ponds of Kiskunság and Hortobágy National Parks located in Hungary is common reed [*Phragmites australis* (Cav.) Trin ex Steudel]. In both areas, saline and alkaline habitats dispose of marsh-like vegetation, but the spreading of floating and attached seaweed-associations can also be observed. Reeds are effective agents of the self purification of these ponds, moreover, form a kind of a buffer zone against the environmental loads coming from the neighboring pastures (Boros 1999). Reeds are promotional in sedimentation and also prevent the enrichment of phytoplankton and moderate eutrophication by decreasing the amount of accessible

nutrients and by shadowing (Amann et al. 1998; Madsen et al. 2001).

Biofilm communities associated with submerged parts of macrophytes can influence the efficiency of self purification processes including the degradation of autochthonous and allochthonous organic matter. Although the decomposition and mineralization of different compounds in nature are determined by complex interactions within microbial communities, our knowledge on the natural catabolic potential has mostly been obtained from the investigation of defined mixed or pure cultures of bacteria (Davey and O'Toole 2000; Stoodley et al. 2002). In the past two decades, application of cultivation-independent molecular biological techniques in microbial ecology has also become effective tools for exploring the diversity of microbial communities (Olsen et al. 1986, Amann et al. 1995).

The aim of the present study was to get insight into the species composition of reed (*Phragmites australis*) periphyton bacterial communities of two Hungarian shallow soda ponds of Kiskunság and Hortobágy National Parks. Different media were applied to reveal the species diversity and metabolic potential of cultivable aerobic organotrophic biofilm bacteria. As a cultivation-independent approach, the phylogenetic diversity of biofilm samples was also investigated by molecular cloning.

Materials and methods

Sampling sites and sample preparation

Kelemen-szék located in Kiskunság National Park (46°47′N; 19°11′E) and Nagy-Vadas located in Hortobágy National Park (47°52′N; 21°40′E) are particularly shallow (average depths 10–40 cm) soda ponds, which periodically dry out completely and have grayish-white lime-sodic water (dominant ions: Na^+, Mg^{2+} and HCO_3^-, CO_3^{2-}). The conductivity and pH values at the time of sampling (in April 2004) were: 1,960 μS cm^{-1} and 9.1 (at Kelemen-szék), 2,240 μS cm^{-1} and 9.08 (at Nagy-Vadas), respectively.

From each sampling site, 10-cm long reed stems (taken from the border of open water) were collected in five replicates from about 10–20 cm below the water surface. Samples were transferred and stored in sterile saline solution (0.89 g l^{-1} NaCl, pH 9) at 4–6°C until laboratory processing was carried out. Biofilm samples were washed from the reed stems into sterile saline solution using sterile brushes (approximately 40–50 cm^2 biofilm per 250 ml saline solution). Cultivation and DNA extraction were made from composite samples prepared from aliquots of the different samples from each sampling site.

Cultivation and characterization of bacterial isolates

From composite samples serial dilutions were made and four different media [King's B, Barrow and Feltham 2003; seawater agar (DSMZ Medium 246); Horikoshi (DSMZ Medium 940) and *Caulobacter* medium (DSMZ Medium 595); http://www.dsmz.de] were used for plating and random isolation, following a 7-day incubation period at 25°C. The pH of the media was adjusted to 9.0.

Colony morphology as well as cell morphology of Hucker's Gram-stained smears was examined. Motility of cells was studied in wet-mount preparations by phase-contrast microscopy. Standard biochemical tests were performed (catalase and oxidase reaction, the oxidative and fermentative utilization of D-glucose, gas production during D-glucose utilization, ammonification, nitrate reduction, methyl red reaction, Voges–Proskauer reaction, H_2S and indole production, urease and phosphatase activity, Simmon's citrate test, casein, gelatine, starch and tween 80 hydrolysis) (Smibert and Krieg 1994). The effect of environmental factors (pH and salt) on growth was investigated by incubation of inoculated nutrient broth (DSMZ Medium 1) for 7 days. The pH range for growth was determined in nutrient broth (supplemented with 50 g l^{-1} NaCl) and pH was adjusted to 7.0–11.0 with KOH at intervals of 1.0 pH units. The NaCl requirement for growth was studied in nutrient broth adjusted to pH 9.0 and supplemented with 0, 5.0, 10.0 and 12.0% (w/v) NaCl. The alteration of the optical density was observed at 600 nm over 1–7 days.

Genomic DNA of the isolates was extracted by PrepMan Ultra (Applied Biosystems) according to the manufacturer's instructions. 16S rDNA was amplified by PCR with 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-TACGGYTACCTTGTTACGACTT-3′) universal eubacterial primer pair (Lane 1991) in a Biometra T Personal thermocycler as described by Borsodi et al. (2007) with the following modification: the first denaturation step was carried out at 98°C for 5 min. PCR products were controlled in 1% agarose gel.

The isolates were grouped by ARDRA (amplified ribosomal DNA restriction analysis) with enzymes *Hin*6I and *Csp*6I (Fermentas) as described by Massol-Deya et al. (1995). Digestion products were separated in 1.5% ethidium-bromide stained agarose gel (Gibco), visualized by UV excitation using Micromax CCD camera, and analyzed by image analyser software (Princetone Instruments).

PCR products were purified and concentrated using the PCR-M™ Clean up System (Viogene). 16S rDNA partial sequence analysis was carried out with the Big Dye Terminator Cycle Sequencing Kit v 3.1 (Applied Biosystems), according to the manufacturer's protocol, using the 519r (5′-GWATTACCGCGGCKGCTG-3′) primer (Lane 1991). Sequencing products were evaluated on a Model 310

Genetic Analyzer (Applied Biosystems). Analysis of sequences and similarity search were carried out using the BLAST algorithm (Altschul et al. 1997) with BLAST (Basic Local Alignment and Search Tool) server of the National Centre for Biotechnology Information. Phylogenetic analysis of the sequences was performed using the Mega 3.1 program package (Kumar et al. 2004). Evolutionary distances were calculated using the Kimura 2 parameter algorithm (Kimura 1980), and a neighbor-joining phylogenetic tree was constructed (Saitou and Nei 1987).

Community DNA isolation and molecular analysis

Composite samples were centrifuged at 5,000×*g* for 10 min. The acquired pellet was used for DNA isolation using FastDNA™ Kit (BIO 101 Inc., CA, USA). Isolated DNA was purified with Geneclean Spin Kit (BIO 101, Inc., CA, USA) according to the manufacturer's instructions.

16S rDNA-fragments of the whole community DNA was amplified by PCR using 27f and 519r primers (Lane 1991) as described by Borsodi et al. (2007) but with a final extension step of 20 min at 72°C. The PCR product was purified and concentrated using the PCR-M™ Clean up System (Viogene) and cloned into *E. coli* JM109 competent cells (Promega) using the pGEM®-T Easy Vector kit (Promega) following the manufacturer's instructions. Plasmids were extracted by boiling a loopful of bacterial cells in 50 µl water (5 min at 98°C) and pelleting the debris by centrifugation (2 min at 15,000×*g*). The supernatant was transferred into fresh tubes. The inserts were further amplified in two PCR steps. The first PCR was carried out using the M13 forward and M13 reverse primers (Stratagene, La Jolla, CA, USA) as in the case of the cultivated isolates, with the following modifications: samples were first denatured at 96°C for 3 min. In the second amplification step, the eubacterial 27f and 519r primers were applied, using the same PCR program as in the case of the first PCR step. Clones were grouped with ARDRA as described in the case of isolates. PCR products of ARDRA group representatives were purified and concentrated using the PCR-M™ Clean up System (Viogene). The sequence analysis of the approximately 500-bp long PCR products was carried out as described above, using the 519r primer (Lane 1991). Sequencing products were evaluated on a Model 310 Genetic Analyzer (Applied Biosystems). All sequences were checked for possible chimeric artifacts by the CHECK_CHIMERA program in the Ribosomal Database Project (RDP) (Maidak et al. 1999). Phylogenetic analysis of the clone sequences was carried out as in the case of the cultivated isolates. The Genbank accession numbers for SSU rRNA gene sequences generated in this study are AM940072–AM940129 for environmental clones.

Results and discussion

The distribution of the 152 isolates among the sampling sites was: 80 from Kelemen-szék (KB) and 72 from Nagy-Vadas (VB). The percentages of positive phenotypic test results are summarized in Table 1. Most of the isolates showed positive result in Gram staining. Facultatively anerobic nitrate reducing as well as urease positive bacteria was present at higher percentage in the KB sample. The bacterial isolates of the KB sample showed higher activities in a number of carbohydrate- and biopolymer utilization tests. Based on low number of positive results in certain tests (anerobic fermentative utilization of D-glucose, Voges–Proskauer and methyl red, Table 1), the cultivable biofilm bacterial communities of Kelemen-szék and Nagy-Vadas could be characterized with an aerobic respiratory metabolism. On the contrary, the results of previous phenotypic investigations on *Phragmites australis* periphyton bacteria in another moderately alkaline soda lake (Lake Velencei, Hungary) referred to the fermentative characteristics of the isolates. The cultivated KB and VB isolates showed lower carbohydrate and biopolymer decomposing activities compared to the test results of Lake Velencei, as well (Borsodi et al. 2007; Rusznyák et al. 2008).

The isolates formed 33 Kelemen-szék (KB) and 20 Nagy-Vadas (VB) ARDRA groups, respectively. From the biofilm of KB and VB, 26 and 18 different bacterial genera and/or species were identified, respectively. The phylotypes of the identified ARDRA group representatives obtained in this study and their closest relatives are shown in Figs. 2 and 3. Altogether 16 isolates, belonging to the genera *Afipia*, *Hydrogenophaga*, *Sphingobacterium*, *Flavobacterium*, *Xanthomonas*, *Halomonas*, *Arhtrobacter*, *Cellulomonas*, *Nesterenkonia*, *Sanguibacter*, *Planococcus* and *Bacillus* showed 97% or less affiliation with known taxa and may represent novel candidate species (Figs. 2, 3), referring to a great uncovered bacterial diversity.

In point of the isolation media, only a few species were isolated exclusively from one medium (e.g., *Acidovorax temperans* from *Caulobacter* medium or *Kocuria rosea* from Horikoshi medium). *Caulobacter* medium was the only one from which representatives of all the six identified bacterial groups (α-, β-, γ-Proteobacteria, Firmicutes, Actinobacteria and the Bacteroidetes group) were isolated. A high number of species belonging to Firmicutes and Actinobacteria group were isolated from seawater agar.

Members of the α-Proteobacteria subgroup were shown to be alkalitolerant and in comparison with isolates belonging to other proteobacterial groups, the majority performed growth up to 10% NaCl concentration (Fig. 1). The largest group of α-Proteobacteria isolates (from both sampling sites) belonged to the species *Paracoccus*

Table 1 Percentile distribution of positive phenotypic test results among the bacterial isolates isolated from reed biofilm samples

	Gram reaction	Oxidase	Catalase	D-Glucose oxidative (24 h)	D-Glucose fermentative (24 h)	Gas production[a]	D-Glucose oxidative (1 week)	Urease activity	Simmons's citrate	D-glucose fermentative (1 week	Phosphatase activity	Ammonification	Hydrolysis of caseine	Motility	Hydrolysis of gelatine	H_2S from cysteine	Indole from tryptophan	Hydrolysis of starch	Reduction of NO_3^- to NO_2^-
Kelemen-szék (80 isolates	58	31	86	15	10	3	26		16		26		55		11		4		18
Nagy-Vadas (72 isolates)	58	29	99	7	10	0	65		14		22		36		19		0		11
	Reduction of NO_3^- to N_2		Reduction of NO_3^- to NH_3		Methyl red reaction	Voges–Proskauer reaction		Aesculin hydrolysis											Hydrolysis of tween 80
Kelemen-szék (80 isolates	9		30		15	6		48	56	48	46	24		50		15		63	
Nagy-Vadas (72 isolates)	0		15		21	1		36	47	38	44	17		65		7		54	

[a] During D-glucose fermentation

carotinifaciens (Figs. 2, 3). These halophilic, Gram negative isolates could be characterized with urease activity, the ability to hydrolyze aesculin, tween 80 and positive result in Simmon's citrate test. The first representatives of the species were isolated from soil samples (Tsubokura et al. 1999). As members of microbial communities in wastewater systems they were found to be present in activated sludge systems, too (Neef et al. 1996).

Members of the genera *Pseudomonas*, *Yersinia*, *Halomonas*, *Xanthomonas*, *Shewanella* and *Acinetobacter* represented the γ-Proteobacteria subgroup (Figs. 2, 3). These isolates from both sampling sites performed optimal growth at 5% NaCl concentration and pH values between 7 and 8 but growth could be observed at pH 10, as well (Fig. 1). The genus *Pseudomonas* was represented by the highest number of identified species within the γ-Proteobacteria among the KB isolates. The species *P. fluorescens*, *P. angulliseptica*, *P. stutzeri and P. marginalis* were represented by our isolates (Figs. 2, 3). These bacteria are characterized with a strictly respiratoric metabolism and are frequently isolated from freshwater and soil samples, as well as from plant-associated microbial communities (Makk et al. 2003; Borsodi et al. 2007). Two isolates (both from KB) were identified as *P. stutzeri*. Due to their catabolic diversity and their ability to tolerate high concentrations of organic matter they also have an important role in the mineralization of organic compounds. One isolate, originating from the VB sample was identified as a member of the genus *Halomonas.* These halotolerant bacteria are known to be able to tolerate a wide range of NaCl concentration (0.1–32%) in their environment. Isolates identified as members of the genus *Halomonas* are frequently isolated from soda lakes worldwide (Ventosa et al. 1998). They are capable of the utilization of a number of organic compounds and show the ability of the accumulation of toxic metal ions (Duckworth et al. 1996, 2000; Vreeland 1999).

From both sampling sites, a number of representatives belonged to different genera of Actinobacteria (*Kocuria*, *Dietzia*, *Sanguibacter*, *Arthrobacter*, *Cellulomonas*, *Plantibacter*, *Streptomyces*, *Microbacterium*, *Nocardiopsis* and *Nesterenkonia*). The majority of these isolates showed growth between 5 and 10% NaCl concentration and pH 8 and 9 (Fig. 1), referring to the adaptation of these isolates to the conditions of the studied ponds. The genus *Nesterenkonia* was represented by the highest number of isolates originating from both sampling sites (Figs. 2, 3). These isolates were fairly inactive in biopolymer utilization tests and were related to the species *N. luteus* and *N. sandarakina*. Representatives of this genus are described as moderately halophilic chemoorganotrophic microorganisms. Another species of the genus, *N. aethiopica* and *N. halobia*, known as alkaliphilic, moderately halophilic

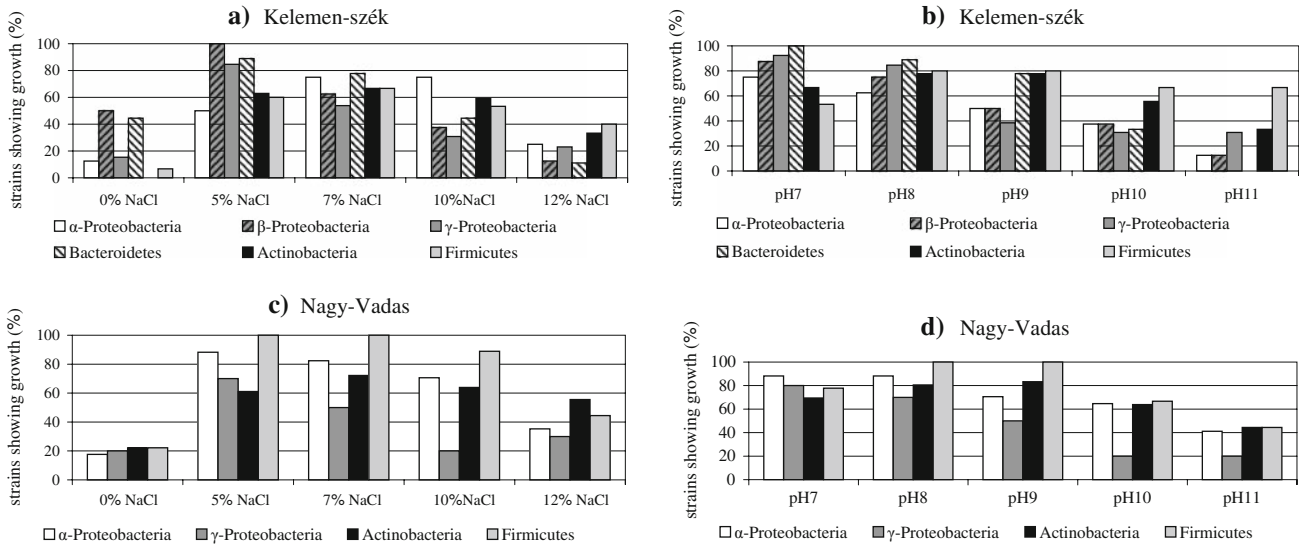

Fig. 1 Results of NaCl (**a**, **c**) and pH tolerance (**b**, **d**) tests of bacterial strains isolated from reed (*Phragmites australis*) periphyton samples taken from Kelemen-szék (**a**, **b**) and Nagy-Vadas (**c**, **d**)

species, was first isolated from a soda lake in Ethiopia (Martins et al. 2001; Delgado et al. 2006). Altogether six isolates from KB, and one isolate from VB were identified as members of the genus *Cellulomonas* (Figs. 2, 3). The partial 16S rRNA sequences of these isolates showed 97% or less affiliation with the species *C. flavigena* and *C. terrae*, possibly representing novel species. These polysaccharase-producing microorganisms can be obtained from various natural environments, for example soil samples (An et al. 2005). The aerobic respiratory *Kocuria rosea* species was represented by two KB isolates (Fig. 2). This species is typical inhabitant of soils and aquatic environments with a broad scale tolerance against environmental factors such as salts, pH and temperature (Borsodi et al. 2007). One isolate originating from the Kelemen-szék sample was identified as the species *Dietzia natronolimnea* (Fig. 2). This isolate was isolated on Horikoshi medium and was able to hydrolyze aesculin and tween 80. *Dietzia* isolates are widely distributed in aquatic habitats, ranging from deep sea sediments to East African soda lakes (Duckworth et al. 1996; Takami et al. 1997; Colquhoun et al. 1998). Isolates of the genus *Dietzia* were also detected as members of cultivable bacterial communities of the rhizomes of declining reed stands in another Hungarian soda lake (Borsodi et al. 2005).

The results of salt- and pH-tolerance tests referred to the alkaliphilic and halotolerant characteristics of the isolates originating from both sampling sites, belonging to the Firmicutes group. More alkaliphilic isolates were present among the KB than among the VB isolates, while an opposite result could be observed in the case of salt tolerance by comparing the two sampling sites (Fig. 1). This phenomenon is reflected in the results of identification: alkalitolerant and alkaliphilic species of the genus *Bacillus* (*B. horikoshii*, *B. licheniformis*) were isolated in greater numbers and formed the largest group within the division in the KB sample, while representatives of the genus *Planococcus*, known as salt-tolerant bacteria were found to be more abundant in the VB sample (Figs. 2, 3). Members of this genus are able to tolerate up to 10% salt concentration and are frequently isolated from seawater (Claus et al. 1999). Most of the isolates identified as *Planococcus* species were able to hydrolyze gelatine. Although the members of the genus *Bacillus* can be characterized with a wide range of decomposing activities, the representative isolates identified as members of this genus showed positive test results only in low number of tests. Several amplicon sequences revealed the presence of this genus studying the diversity of alkaliphiles in a Kenyan soda lake (Rees et al. 2004). The Gram positive, endospore-forming *Bacillus* species are frequently isolated and detected as predominant species in freshwater habitats, as well (Borsodi et al. 1998, 2003).

Bacillus and *Pseudomonas* species were also found to be abundant members of reed biofilm and rhizome samples taken from Lake Velencei (Borsodi et al. 2007; Micsinai et al. 2003). At the same time, members of the genera *Paracoccus*, *Halomonas*, *Planococcus*, *Dietzia* and *Nesterenkonia* were only represented by isolates originating from Kelemen-szék and Nagy-Vadas presumably due to the higher alkalinity and salinity of these two sampling sites.

Contrary to the phylogenetic lineages discussed above, representatives of the groups of β-Proteobacteria and Bacteroidetes were detected only in the biofilm sample from Kelemen-szék (Fig. 2). Two isolates were members

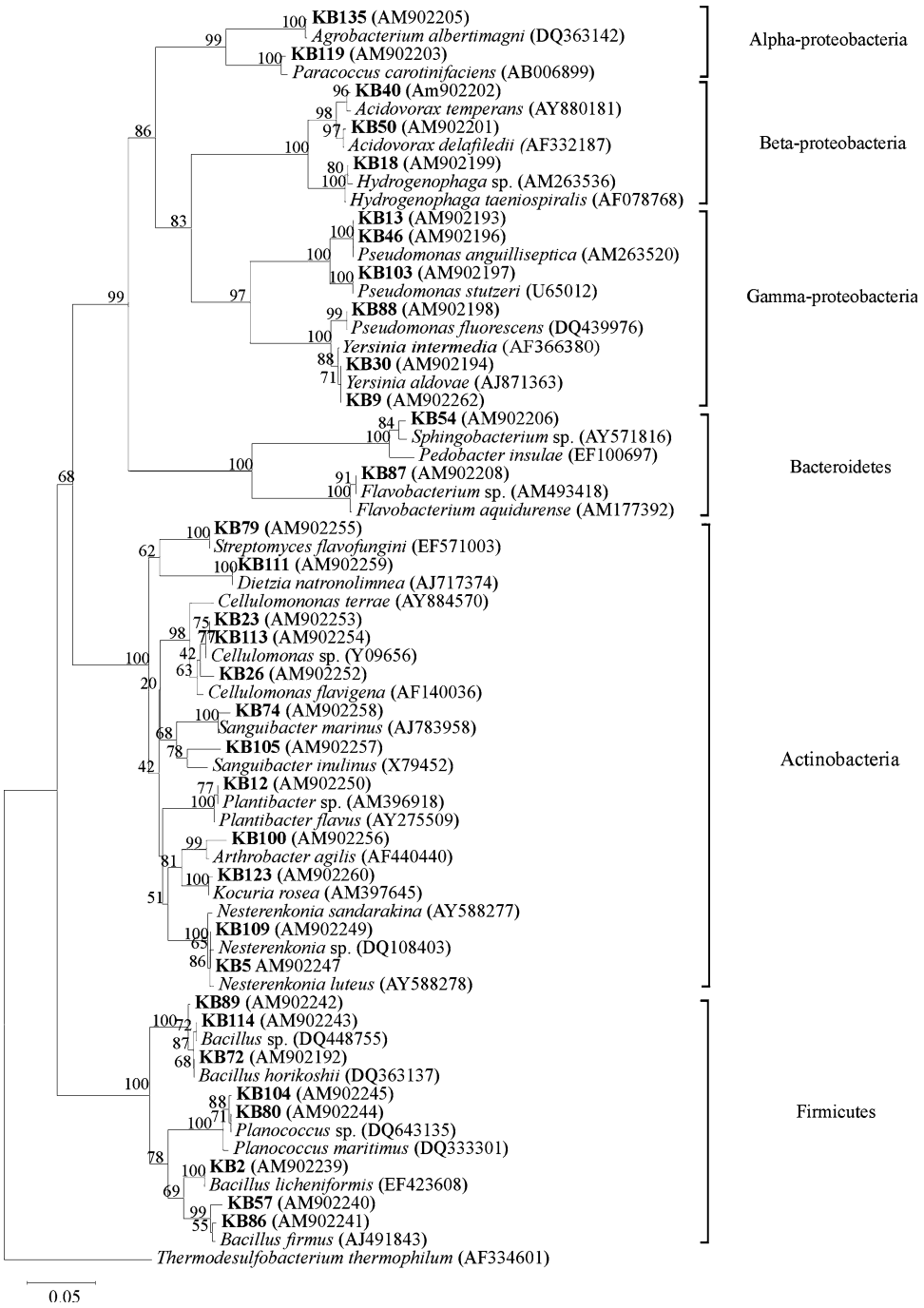

Fig. 2 16S rDNA sequence based neighbor-joining phylogenetic tree of bacterial strains isolated from reed (*Phragmites australis*) periphyton samples originating from Kelemen-szék

of the genus *Hydrogenophaga* and altogether six isolates were identified as *Acidovorax delafieldii* and *A. temperans*. These bacteria are common inhabitants of aquatic environments and because of their metabolic properties they are important members of activated sludge microbial communities, as well (Schulze et al. 1999; Kampfer et al. 1996; Kampfer et al. 1996, 2006). The species *Flavobacterium* and *Sphingobacterium* were represented by nine isolates. Representatives of these genera are also common members of freshwater environments (Jooste and Hugo 1999).

In order to complement the cultivation-based investigations, bacterial diversity of biofilm communities from the two sampling sites was also investigated by the cultivation-independent cloning approach. The screening of 129 bacterial clones from Kelemen-szék (KBC) and 158 from Nagy-Vadas (VBC) resulted in 30 and 28 ARDRA-groups, respectively (Table 2). Chimeric sequences and inserts identified as diatom chloroplast or cyanobacterium sequences were omitted. The results of cloning revealed a Gram-negative dominated diversity within the reed

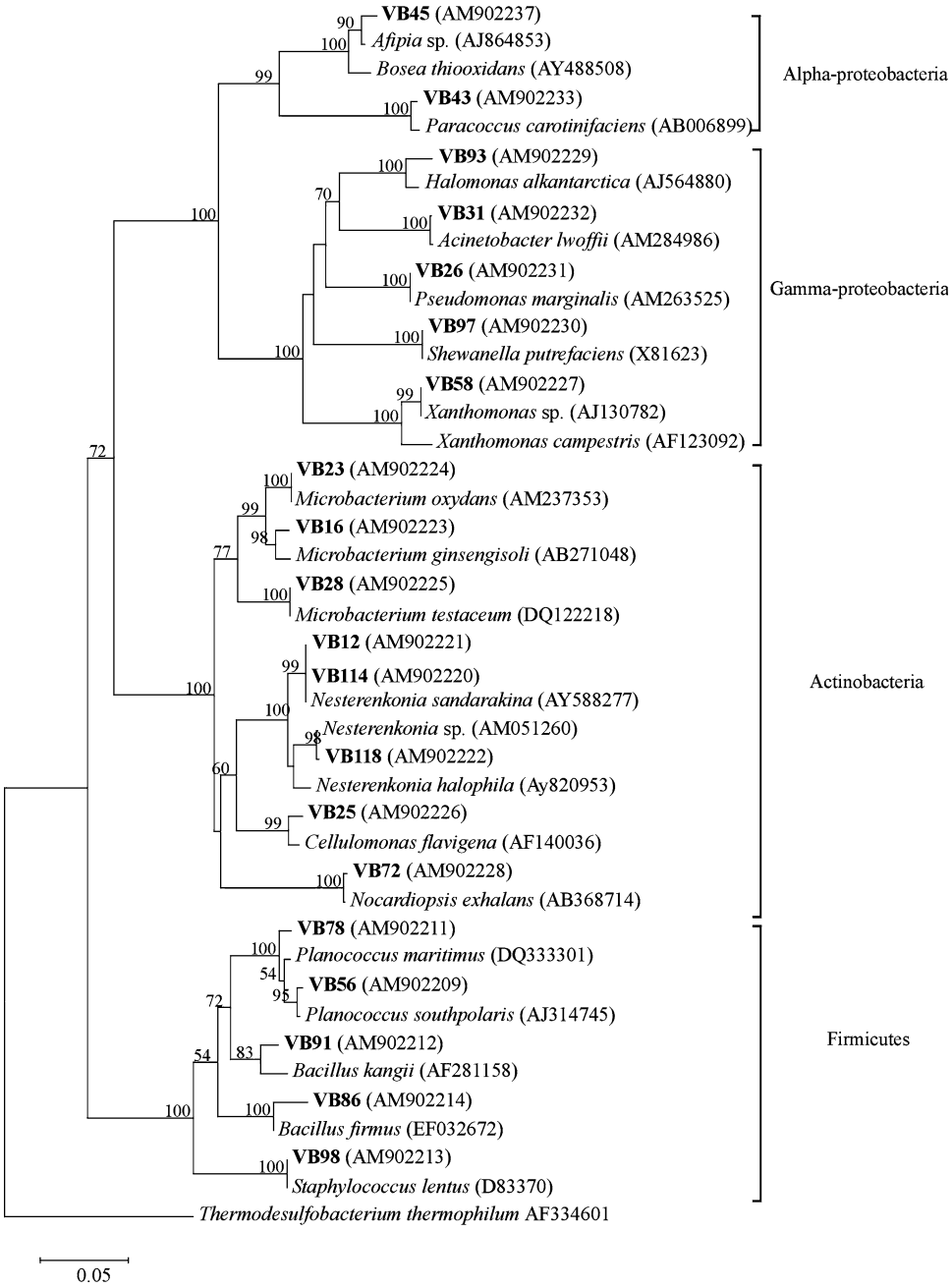

Fig. 3 16S rDNA sequence based neighbor-joining phylogenetic tree of bacterial strains isolated from reed (*Phragmites australis*) periphyton samples originating from Nagy-Vadas

periphyton bacterial communities of both sites. The Firmicutes group was not, and the Actinobacteria group was represented only by three KBC clones. Among the VBC clones no Gram positive representative was detected. Furthermore, the percentage of sequences showing affiliation with uncultured bacterial clones was 30% in Kelemenszék and 22% in the Nagy-Vadas, referring to a great and yet not cultured diversity of bacterial communities in the biofilm samples.

More than 50% of the clones represented the three proteobacterial subgroups (Table 2). Altogether three different genera within the γ-Proteobacteria were found to be present in the samples from both sampling sites. Such Gram-negative, aerobic, heterotrophic, non-fermentative bacteria are associated with freshwater environments (*Aquimonas voraii*), marine habitats (*Rheinheimera* sp.) and were previously found in soil samples (*Cellvibrio gandavensis*) (Mergaert et al. 2003; Romanenko et al. 2003; Saha et al. 2005).

More than 30% of bacterial clones belonged to the Bacteroidetes group (the genera *Dyadobacter*, *Paludibacter*, *Gelidibacter*, *Flavobacterium*, *Sphingobacterium*, *Fluviicola* and *Algoriphagus*) in the case of both clone libraries. The latter fact indicates that there is currently a

Table 2 Phylogenetic affiliation of partial 16S rDNA clone sequences obtained from biofilm samples taken in Kelemen-szék and Nagy-Vadas

	Number of clones	Closest relative	Sequence similarity[a]
Kelemen-szék			
α-Proteobacteria	3	*Agrobacterium* sp. (AY826532)	98% (406/403)
	6	*Agrobacterium sanguineum* (AB062106)	99% (410/414)
	3	Uncultured α-proteobacterium clone (AF236002)	97% (404/415)
β-Proteobacteria	2	*Hydrogenophaga atypica* (AJ585992)	97% (446/459)
	2	*Hydrogenophaga taeniospiralis* (AY771764)	98% (427/434)
	3	*Dechloromonas denitrificans* (AJ318917)	98% (448/454)
	3	*Dechloromonas hortensis* (AY277621)	98% (459/466)
	4	*Pelomonas saccharophila* (AB021407)	98% (424/432)
	2	Uncultured Comamonadaceae bacterium clone (EF370588)	97% (428/440)
	2	Uncultured bacterium clone (DQ066963)	97% (428/441)
γ-Proteobacteria	5	*Rheinheimera* sp. (EF575565)	98% (441/449)
		Rheinheimera aquimaris (EF076758)	97% (347/357)
	1	*Rheinheimera chironomi* (DQ298025)	98% (419/425)
	4	*Rheinheimera chironomi* (DQ298025)	98% (398/404)
	1	*Rheinheimera* sp. (EF575565)	98% (406/414)
	4	Uncultured γ-proteobacterium clone (AF141439)	97% (394/404)
	4	*Cellvibrio gandavensis* (AJ289162)	99% (413/414)
	3	*Aquimonas voraii* (AY544768)	98% (429/435)
Bacteroidetes	4	*Dyadobacter fermentans* (AF137029)	97% (370/379)
	4	*Palucidibacter propionicigenes* (AB078842)	98% (423/429)
	6	Uncultured Bacteroidetes sp. Clone (AJ534686)	94% (403/425)
	2	*Flavobacterium hibernum* (AJ251068)	98% (299/305)
	2	*Gelidibacter* sp. (AY259512)	97% (427/437)
	3	Uncultured CFB group bacterium clone (AY043735)	98% (406/414)
	1	Uncultured bacterium clone (AB297423)	97% (429/442)
	4	*Sphingobacterium* sp. (AY571816)	98% (439/447)
		Pedobacter insulae (EF100697)	97% (433/446)
	3	*Flavobacterium* sp. (AJ876670)	97% (447/457)
	4	Uncultured Flavobacteria bacterium clone (AM279209)	97% (450/462)
	1	*Flavobacterium sp.* (AJ876670)	97% (446/458)
Firmicutes (high G + C Gram positives)	3	*Arthrobacter agilis* (EF010549)	97% (367/375)
Fibrobacteres group	4	Unidentified eubacterium clone (AJ232823)	98% (442/448)
Nagy-Vadas			
α-Proteobacteria	6	*Sphingomonas jaspsi* (AB264131)	98% (382/389)
	6	*Agrobacterium albertimagni* (DQ363142)	98% (428/436)
	4	*Agrobacterium sanguineum* (DQ363139)	99% (401/402)
	4	*Agrobacterium albertimagni* (DQ363142)	97% (405/417)
	7	*Paracoccus* sp. (AJ309981)	97% (422/434)
		Paracoccus carotinifaciens (AB006899)	96% (419/432)
	4	Uncultured α-proteobacterium bacterium clone (AJ888555)	97% (389/400)
β-Proteobacteria	4	*Hydrogenophaga teinospiralis* (AF078768)	98% (396/402)
	2	*Hydrogenophaga defluvii* (AJ585993)	98% (470/476)
	3	Uncultured bacterium clone (AY546509)	97% (401/410)
	4	*Malikia spinosa* (AB021387)	96% (391/404)
	6	*Polaromonas aquatica* (AM039830)	98% (384/390)
	3	*Aquabacterium honkongensis* (DQ489306)	98% (411/418)

Table 2 continued

	Number of clones	Closest relative	Sequence similarity[a]
	3	*Ideonella* sp. (DQ664241)	97% (457/471)
	1	Uncultured *Ideonella* sp. bacterium clone (AY435508)	98% (463/472)
	4	*Ideonella* sp. (AB211233)	94% (402/425)
	3	*Hydrogenophaga atypica* (AJ585992)	99% (467/471)
	1	Uncultured β-proteobacterium bacterium clone (AY678517)	97% (468/479)
γ-Proteobacteria	5	Uncultured γ-proteobacterium bacterium clone (AJ810621)	98% (392/399)
	3	*Cellvibrio vulgaris* (AF448513)	96% (406/419)
	5	*Aquimonas voraii* (AY544768)	99% (440/443)
	3	*Rheinheimera* sp. (EF575565)	100% (434/434)
		Rheinheimera chironomi (DQ298025)	97% (421/430)
Bacteroidetes	11	*Flavobacterium columnare* (AJ491824)	99% (419/421)
	3	*Flavobacterium filum* (DQ372981)	98% (410/417)
	2	*Flavobacterium kamogawaensis* (AB275999)	92% (427/463)
	3	*Fluviicola taffensis* (AF493694)	97% (407/418)
	5	Uncultured Bacteroidetes sp. bacterium clone (DQ463716)	97% (433/443)
	16	*Algoriphagus halophilus* (AY264839)	96% (337/350)
Chloroflexi group	2	Uncultured Chloroflexi bacterium clone (AY921893)	97% (420/432)

[a] Numbers in parenthesis: length of sequence used for calculation of sequence similarity with closest relative

lack of cultured bacteria belonging to the Bacteroidetes group in the sequence databases, suggesting that further studies are needed to provide more information about the diversity of the Bacteroidetes division in natural environments (O'Sullivan et al. 2002).

Some of the clone sequences were obtained only in either of the two reed periphyton communities and verified the presence of bacteria originally isolated from various environmental sources, such as freshwaters (e.g., *Malikia* sp., *Aquabacterium* sp., *Fluviicola taffensis*), marine sediments (*Algoriphagus halophilus*), soil samples (e.g., *Dechloromonas hortensis*, *Paludibacter propiocigenes*) or plant-associated communities (*Dyadobacter fermentans*) (Chelius and Triplett 2000; Brümmer et al. 2003; Nedashkovskaya et al. 2004; Spring et al. 2005; O'Sullivan et al. 2005; Wolterink et al. 2005; Ueki et al. 2006). The sequence analysis of an ARDRA group representative of four clones proved the presence of the Fibrobacteres group in the biofilm bacterial communities of Kelemen-szék, and the sequence of one representative clone from Nagy-Vadas was affiliated with an uncultured Chloroflexi bacterium clone, as well.

Members of altogether six different genera (*Paracoccus, Agrobacterium, Hydrogenophaga*, *Flavobacterium, Sphingobacterium* and *Arthrobacter*) were detected by both techniques. The species *Agrobacterium albertimagni* was represented by isolates from the Kelemen-szék and clones from Nagy-Vadas, while *Agrobacterium sanguineum* was found in both samples only by the sequence analysis of representative clones. Some members of the genus *Rhizobium* (*Agrobacterium*), characterized with an aerobic respiratory metabolism are known as pathogens of dicotyledonous plants. Their role in the case of monocotyledonous plants is yet not clarified; most probably they are able to participate in associative nitrogen fixation (Eady 2001). Although a great diversity of β-Proteobacteria and the Bacteroidetes group was detected by cloning, only the genera *Hydrogenophaga*, *Flavobacterium* and *Sphingobacterium* were confirmed both by cloning and cultivation. Representatives of the genus *Flavobacterium* and *Sphingobacterium* are shown to be sensitive to the cultivation conditions, e.g., salt concentration and organic matter content (Jooste and Hugo 1999; O'Sullivan et al. 2002), this may be a reason that members of these genera were found to be present by low number of isolates, originating only from the Kelemen-szék biofilm sample. Among Gram positives, the genus *Arthrobacter* was the only taxon which was represented by bacterial isolates as well as by clones of Kelemen-szék. Similar contrast of the results of cultivation, referring to a Gram-positive dominance and the sequence analysis of clones, revealing a Gram-negative dominated bacterial diversity could be observed by Ma et al. (2004) during the investigations of the bacterial diversity of the Inner Mongolian Baer soda lake. The controversial results may arise from the different selectivity of the two approaches. Gram-positive microorganisms, especially the ones able to produce endospores, are known to be easier to cultivate and maintain under laboratory conditions than Gram-negatives. On the other hand, the possible preferential DNA extraction

procedures from various environmental samples can lead to an overestimation of the abundance of Gram-negative bacteria within the studied microbial community, if solely DNA-based methods are applied for investigations.

In conclusion, cultivation based examinations revealed mainly aerobic oxidative metabolism of the isolates from both sampling sites but higher activities in a number of phenotypic tests were detected among the isolates from the reed periphyton of Kelemen-szék. The results of salt and pH tolerance tests revealed principally alkaliphilic and alkalitolerant as well as moderately halophilic bacteria from the biofilm of both sampling sites, referring to the adaptation of these bacteria to the studied environments. The 16S rRNA based identification revealed a definite Gram-positive dominance of the cultivated isolates while unambiguous Gram-negative dominance of the clones could be observed from both sampling sites. Reed periphyton bacterial communities from the more particularly fluctuating soda pond (Kelemen-szék) can be characterized by a wider phylogenetic and metabolic diversity. The dissimilar results gained by the two methods drive attention to the need of application of the polyphasic approach in microbial ecological investigations. Cultivation-independent methods that are based solely on phylogenetic sequence information are not adequate to reveal the metabolism of microorganisms in a given environment, nevertheless can expose a not yet cultured, hidden diversity as it was in the case of our study, as well. The cultivation-based approach alone is neither appropriate for the description of biodiversity in the case of such a complex community as a biofilm. However, similarly our findings in this study, traditional cultivation based methods have a great importance in bacteriological research providing the opportunity of detailed investigations of potentially novel species of bacterial isolates under laboratory conditions.

Acknowledgments This research was supported by the Hungarian Scientific Research Fund (OTKA) Grant T038021.

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