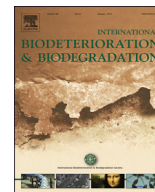




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Diversity and efficiency of anthracene-degrading bacteria isolated from a denitrifying activated sludge system treating municipal wastewater



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ABSTRACT

Although several attempts have been performed to isolate PAHs-degrading bacteria, the composition of sewage activated sludge microbiota capable of degrading PAHs still remains unexplored. In this study, anthracene-degrading bacteria were isolated from the activated sludge of an intermittently aerated and pulse fed bioreactor treating municipal wastewater and operating under high organic loading conditions (F/M of 0.468 kg BOD₅ kg⁻¹ VSS d⁻¹). After two successive enrichment steps, a total of 51 bacterial strains growing on anthracene-containing medium were isolated. Based on 16S rRNA gene sequencing, the bacterial strains isolated were classified into 17 operational taxonomic units and their members were phylogenetically related to known denitrifying species. The majority of the isolates were anthracene-degraders belonging to *Gammaproteobacteria* (30.4% of the isolates), *Actinobacteria* (28.3%), *Alphaproteobacteria* (21.7%) and *Betaproteobacteria* (19.6%). The greatest anthracene degradation was achieved by bacterial isolates associated with the species *Microbacterium arabinogalactanolyticum*, *Shinella zoogloeoides* and *Alicyclophilus denitrificans* (61–69% degradation efficiency), followed by strains related to *Paracoccus huijuniae* (39%). Indeed, this is the first report on effective PAH degradation by members of the genera *Alicyclophilus* and *Shinella*. In conclusion, a broad anthracene-degrading bacterial community was identified, indicating the multifunctionality and versatility of municipal activated sludge to cope with a wide variety of micro-pollutants, including PAHs.

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are hydrocarbons with two or more fused aromatic rings in straight-lined, angled and group formations. They are produced during incomplete breakdown of organic compounds at high temperatures and subsequent recombination of their residual thermal by-products (Haritash and Kaushik, 2009). PAHs are considered as an

important group of organic pollutants, generated from either natural or anthropogenic sources. PAHs can be released from natural sources, such as vegetation fires and volcanic vent materials, while fossil fuel and incineration emissions, coal tar, lubricants and oil spills are among the anthropogenic causes of PAHs (Lima et al., 2005). However, their discharge to the atmosphere is mainly anthropogenic, as a consequence of an increased fossil fuel demand (Lima et al., 2005; Haritash and Kaushik, 2009).

Modern societies have an increased concern of the environmental impact of PAHs emitted, due to their widespread occurrence and high bioaccumulation potential and carcinogenic activity. The U.S. EPA has defined 16 PAHs as high priority pollutants, while six of them are considered as potential carcinogenic compounds (Bojes and Pope, 2007). Although volatilization and photochemical oxidation have been applied for the removal of PAHs, microbial decomposition is considered as the main approach for their degradation (Yuan et al., 2001). Microbial degradation of PAHs results in less complex metabolites and often in their complete

Abbreviations: ANOVA, analysis of variance; BOD₅, 5-day biochemical oxygen demand; BNR, biological nutrient removal; CoA, coenzyme A; COD, chemical oxygen demand; MLSS, mixed liquor suspended solids; MLVSS, mixed liquor volatile suspended solids; NCBI, National Center for Biotechnology Information; PAHs, polycyclic aromatic hydrocarbons; PCA, plate count agar; PCR, polymerase chain reaction; RDP, Ribosomal DNA Project; SPE, solid phase extraction; SRT, sludge retention time; SS, suspended solids; U.S. EPA, U.S. Environmental Protection Agency; WWTP, Wastewater Treatment Plant.

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mineralization, with CO₂ and CH₄ as the main end-products of oxidative and methanogenic metabolism, respectively. The rate of degradation depends on a large number of factors, such as PAHs structure, growth temperature and pH, salinity level, oxygen concentration and acclimatization conditions (Haritash and Kaushik, 2009). A certain number of microorganisms (mostly bacteria and fungi) are capable of degrading various PAHs compounds under both aerobic and anaerobic conditions (Haritash and Kaushik, 2009; Fuchs et al., 2011). Most of them have been obtained from PAHs-contaminated sediments and soils (Peng et al., 2008), although microbial degraders from activated sludge have been rarely isolated.

Different metabolic mechanisms can be adopted by microorganisms to bioremediate PAHs-contaminated environments. Aerobic degradation of PAHs is mainly carried out through the initial attack of dioxygenases to the phenyl ring, resulting in the formation of catechol, although alternative metabolic pathways, in which ring cleavage leads to epoxide via CoA thioesters activation and ring-opening hydrolysis, have been identified (Fuchs et al., 2011). Microbial decomposition of catechol to metabolites entering the tricarboxylic acid cycle is further achieved via the action of 1,2- or 2,3-catechol dioxygenases (ortho- or meta-cleavage respectively), allowing the complete mineralization of this aromatic hydrocarbons series. Besides to assimilatory pathways, bioaccumulation is an alternative strategy for microbial cells to cope with PAHs toxicity, since the latter are stored to microbial lipid fraction and might be transformed to more water-soluble molecules (Subashchandra et al., 2013). Co-metabolism can enhance PAHs degradation, whereas indigenous bioaugmentation affects pollutant bioavailability (Hwang and Cutright, 2002).

Although attention has been paid to the isolation of microbial degraders from PAHs-contaminated soils, limited attempts have been made in order to isolate microorganisms capable of decreasing PAHs concentration in wastewater treatment plants treating municipal wastewaters. In these studies, single PAH-degrading strains associated with the genera *Pseudomonas* and *Sphingomonas* were isolated (Ma et al., 2005; Avramova et al., 2008). However, the cultured PAHs-degrading communities in activated sludge systems treating municipal wastewaters still remained uncovered.

In this research work, it is attempted for the first time to identify by molecular techniques the cultured anthracene-degrading population in the activated sludge of a BNR system treating municipal wastewater and to determine their anthracene degradation potential through the enforcement of an anthracene enrichment procedure and the implementation of solid phase extraction (SPE) and high performance liquid chromatography (HPLC) techniques.

Materials and methods

Bioreactor design and physicochemical analysis

An intermittently aerated and pulse fed pilot-scale BNR system was used for nutrients removal from municipal wastewater obtained from the WWTP of Xanthi, North Greece. The system consisted of a 9 L influent settling tank, a main bioreactor of 45 L working volume and a 8.5 L final clarifier, thereby part of biomass was directed to the bioreactor in order to retain an SRT of 15 days (Melidis et al., 2014). Alternating aerobic-anoxic phases of 25 min/35 min respectively were used to achieve denitrification. Influent feeding was performed at the beginning of the anoxic phase (within the first 5 min). Determination of NH₄⁺-N, BOD₅, COD, MLSS, MLVSS, NO₃⁻-N and SS concentrations was performed according to Clesceri et al. (1998).

Enrichment, isolation procedure and degradation potential of anthracene-degrading bacteria

Enrichment procedure included addition of 10 ml mixed liquor (sonicated on ice for 60 s at 50% duty level and 50 W output power) in 90 ml of liquid medium, consisting of 10 mg L⁻¹ crystalline anthracene, 12.5 mM NH₄Cl, 12.5 mM KNO₃, 7.35 mM KH₂PO₄, 5.75 mM K₂HPO₄, 0.81 mM MgSO₄, 0.13 mM CaCl₂ and two drops of saturated FeCl₂. After 10-days incubation period at 25 °C, 1 ml of the exhausted culture was transferred to 99 ml of the abovementioned (fresh) liquid medium and further incubated for 10 days. In the initial inoculum (mixed liquor), bacterial population was enumerated using plate count agar (PCA), while the anthracene-growing population was counted at each enrichment step using the anthracene-based medium described above in the presence of 17 g L⁻¹ agar with the modification that anthracene solution was diluted in acetone, spread on agar plate and allowed to dry prior to use.

The anthracene concentration accumulated in the growing cells was determined at the end of the second enrichment step through 0.2 μm micro-filtration and hexane extraction of the filtered biomass.

To isolate and enumerate the anthracene-growing population in the mixed liquor of the intermittently aerated and fed bioreactor treating municipal wastewater, tenfold dilution series were accomplished and 0.1 ml of each suspension were aliquoted into three anthracene-containing agar plates. Colonies were enumerated after 2 weeks incubation period and the anthracene-growing population was expressed as colony forming units (cfu) per ml mixed liquor or inoculated liquid medium. All bacterial colonies from one of the three plates in the 10⁻⁴ dilution were obtained.

Degradation of anthracene by the bacterial strains isolated was examined in 100 ml of the basal medium described above. The inoculated cultures were incubated for 5 days at 25 °C and 120 rpm.

Solid phase extraction

Solid phase extraction of anthracene-containing liquid media was carried out by affixing SPE cartridges (Resprep-C8-47 SPE Disks

Table 1

Operational and physicochemical parameters of the intermittently aerated and pulse fed pilot-scale bioreactor treating municipal wastewater at the time of enrichment procedure.

Parameter	Value
HRT (d)	0.47
V (m ³)	0.045
Q (m ³ d ⁻¹)	0.096
Period of feeding cycling (h)	1
Anoxic/oxic phase (min/min)	35/25
F/M (kg BOD ₅ kg ⁻¹ VSS d ⁻¹)	0.468
tCOD _{in} (mg L ⁻¹)	715 ± 13
tCOD _{ef} (mg L ⁻¹)	67.2 ± 13.6
BOD _{in} (mg L ⁻¹)	450 ± 42
BOD _{ef} (mg L ⁻¹)	29.5 ± 0.7
MLSS (g L ⁻¹)	2.42 ± 0.88
MLVSS (g L ⁻¹)	2.05 ± 0.67
SS _{in} (mg L ⁻¹)	298 ± 10
SS _{ef} (mg L ⁻¹)	30.5 ± 6.4
SRT (d)	15
SVI (ml g ⁻¹)	115 ± 10
NH ₄ ⁺ -N _{in} (mg L ⁻¹)	67.7 ± 14.6
NH ₄ ⁺ -N _{ef} (mg L ⁻¹)	1.7 ± 0.5
NO ₃ ⁻ -N _{ef} (mg L ⁻¹)	2.0 ± 0.1
Anthracene _{in} (ng L ⁻¹)	130 ± 34
Anthracene _{ef} (ng L ⁻¹)	0 ^a

^a Below the detection limit.

fitted to Diskcover-47 Extraction Disk Holder - both purchased by Restek) to Visiprep™ 12- Port Vacuum Manifold (Sigma), following the US EPA 550.1 method (Hodgeson, 1990). The cartridges were conditioned by successively adding equal quantities (5 ml) of dichloromethane, methanol and water. After sample elution, anthracene was recovered by adding 4 ml acetonitrile and 2 × 4 ml dichloromethane and the eluate was dried under a gentle N₂

stream. The residue was dissolved in appropriate volume of methanol and filtered prior to injection into HPLC column.

HPLC analysis

Anthracene concentrations were determined using a DIONEX high-performance liquid chromatography system (Dionex, USA).

Table 2

Operational taxonomic units and closest phylogenetic relatives of anthracene-degrading bacteria isolated from the activated sludge of the intermittently aerated and pulse fed pilot-scale bioreactor treating municipal wastewater.

OTUs	Isolates (accession numbers)	Closest phylogenetic relatives	Similarity (%)	Habitat	Accession no – reference
1	ADA-6, ADC-4, ADC-11, ADC-12, ADC-23A, ADC-23B, ADC-25A, ADC-33, ADC-37 (KM210226–KM210234)	Strain A3/ <i>Pseudomonas citronellolis</i> ADP (DSM 11735) [/] Strain wust-c Strain G4/ <i>Pseudomonas citronellolis</i> DSM 50332 ^T	99.5–99.9/ 99.5–99.9/ 99.5–99.9/ 99.4–99.8/ 99.3–99.7	3-Chlorobenzoate degrader/ Atrazine degrader/ Activated sludge/ Petroleum-contaminated soils/ Soil ^a	Y13246/ Mandelbaum et al. (1995)/ JN180124/ Ozaki et al. (2006)/ NR_026533
2	ADA-1A, ADA-1B, ADA-3A, ADA-3B, ADA-8B, ADC-4A (KM210235–KM210240)	Strain JCM 9635/ Strain PLL-3/ Strain 4APF/ <i>M. arabinogalactanolyticum</i> DSM 8611 ^T	99.9–100/ 99.8–99.9/ 99.7–99.9/ 99.7–99.9	Soil, Japan ^b / <i>p</i> -Nitrophenol-degrader/ 4-Aminopyridine degrader/ Hospital material	AB563788/ EU127453/ AB695354/ NR_044932
3	ADC-3, ADC-10, ADC-10A, ADC-10B, ADC-18 (KM210241–KM210245)	<i>Shinella zoogloeoides</i> IAM 12669 ^T	99.8	Organically polluted freshwater and wastewaters	Unz (1984)
4	ADC-14, ADC-16, ADC-21, ADC-24, ADC-26A (KM210246–KM210250)	Strain R-24611/ <i>Alicyclophilus denitrificans</i> K601 ^T	99.9–100/ 99.7	Denitrifying activated sludge/ Cyclohexanol-degrading denitrifier	Heylen et al. (2006)/ NR_025510
5	ADA-2, ADC-2, ADC-8, ADC-9, ADC-20 (KM210251–KM210255)	Strain Z25/ <i>Rhodococcus aetherivorans</i> NBRC 106353/ <i>Rhodococcus aetherivorans</i> DSM 44752 ^T / <i>Rhodococcus ruber</i> DSM 43338 ^T	99.6–100/ 99.5–99.9/ 99.4–99.9/ 99.6–100	Biosurfactant producer/ Soil, Japan/ Activated sludge ^c / Soil	FJ752527/ AB546298/ NR_118619/ Goodfellow (1986)
6	ADA-4B, ADA-8A, ADC-4B, ADC-13 (KM210256–KM210259)	<i>Stenotrophomonas</i> sp. LMG 19833/ <i>S. acidaminiphila</i> ATCC 700916 ^T	99.9–100/ 99.7–99.8	Denitrifying continuous upflow fixed-bed reactor/ UASB reactor	AJ300772/ NR_025104
7	ADB-32, ADC-1, ADC-15A (KM210260–KM210262)	Stain R-24650/ <i>Paracoccus huijuniae</i> FLN-7 ^T	99.5–99.9/ 99.6–99.9	Denitrifying activated sludge/ Amide pesticide-degrader from activated sludge	Heylen et al. (2006)/ NR_108224
8	ADC-27, ADC-27B (KM210263–KM210264)	Clone 7-41/ Strain TG26/ <i>Castellaniella daejeonensis</i> MJ06 ^T / <i>Castellaniella defragrans</i> 54Pin ^T	100/ 99.7/ 98.6/ 98.4	Drinking water distribution system/ Denitrifier and phenol-degrader/ Soil/ Grow on monoterpenes and nitrate	JQ923874/ AF384193/ NR_117260/ NR_025280
9	ADA-7, ADC-7 (KM210265–KM210266)	strain 13635E/ <i>Microbacterium xylanilyticum</i> S3-E ^T	98.8/ 98.4	Marine sediment/ Xylan degrader	EU741115/ NR_042350
10	ADA-5, ADC-27A (KM210267–KM210268)	strain MTR32A/ <i>Shinella fusca</i> DC-196 ^T	99.7–99.8/ 99.7–99.8	Mine tailings/ Compost	DQ507209/ NR_116889
11	ADC-6, ADC-31 (KM210269–KM210270)	Strain MT-E3/ <i>Achromobacter dolens</i> LMG 26840 ^T / <i>Achromobacter anxifer</i> LMG 26857 ^T / <i>Achromobacter denitrificans</i> DSM 30026 ^T	99.7/ 99.5/ 99.5/ 99.5	Industrial wastewater / Human sputum/ Human sputum/ Soil ^d	Toups et al. (2010)/ Vandamme et al. (2013)/ Vandamme et al. (2013)/ NR_042021
12	ADB-5 (KM210271)	Clone B4/ <i>Pseudoxanthomonas mexicana</i> AMX 26B ^T	99.8/ 99.8	Wastewater/ UASB reactor sludge	HM007535/ NR_025105
13	ADC-22 (KM210272)	clone UPB-1/ <i>Aquamicrobium lusatiense</i> DSM 11099 ^T	99.9/ 99.9	Biofilter for landfill odor treatment/ Chloroheanol-degrader	JX534186/ NR_025312
14	ADB-34 (KM210273)	Strain DDB001/ <i>Devosia insulae</i> DS-56 ^T	99.9/ 96.0	Soil/ Soil	JX392051/ NR_044036
15	ADC-19B (KM210274)	Clone AnSal-08/ <i>Mesorhizobium tamadayense</i> Ala-3 ^T	100/ 98.7	Anammox sludge/ Rhizobium	AB434260/ NR_115048
16	ADB-19A (KM210275)	<i>Oligotropha carboxidovorans</i> OM5 ^T	100	Wastewater	Paul et al. (2008)
17	ADC-38 (KM210276)	Strain SW102/ <i>Flexivirga alba</i> ST13 ^T	98.3/ 98.3	Soil/ Hexavalent chromium reducer	HQ418230/ NR_113034

^a <http://www.dsmz.de/catalogues/details/culture/DSM-50332>.

^b <http://www.jcm.riken.jp/>.

^c <http://www.dsmz.de/catalogues/details/culture/DSM-44752>.

^d <http://www.dsmz.de/catalogues/details/culture/DSM-30026>.

The system consisted of a DIONEX P680A gradient pump supported with a Rheodyne 8125 injector and a 20 μ l loop. The mobile phase was acetonitrile and water, which set at a flow rate of 1 ml/min. The gradient elution scheme initiated with 50% acetonitrile and moved linearly to 100% in a run period of 25 min, following the Restek instructions for Pinnacle II PAH HPLC column (based on US EPA method 8310, 1986). The Restek Pinnacle II PAH C18 reverse phase column (250 mm \times 4.6 mm, particle size 4 μ m, pore size 110 Å) was fitted in the system and operated at 25 °C. Anthracene was determined in a UV/VIS 170U detector (Dionex, USA) at 254 nm and quantification was carried out against external standards (Sigma).

Genomic DNA extraction and 16S rRNA gene amplification

Genomic DNA from the bacterial strains isolated was extracted using the “GF-1 Bacterial DNA Extraction” kit (Vivantis, Malaysia). A reaction mixture (50 mL) consisting of 1 μ L (50 ng μ L⁻¹) genomic DNA, 10 \times PCR buffer, 50 mM MgCl₂, 10 mM each dNTP, 50 μ M primers pA (5'-AGA GTT TGA TCC TGG CTC AG-3') and pH (5'-AAG GAG GTG ATC CAG CCG CA-3') and 2.5 U DNA polymerase (Maximo Taq DNA Polymerase, Geneon, Germany) was used for PCR amplification. The amplification of 16S rRNA gene was carried out in a Gradient PCR cyler (TaKaRa, Japan) under an initial denaturation stage of 2 min at 94 °C, followed by 35 cycles of 30 s denaturation at 94 °C, 30 s primer annealing at 52 °C, and 75 s DNA chain extension at 72 °C. PCR reaction was terminated by a 5 min chain elongation stage at 72 °C.

Cloning and 16S rRNA gene sequencing

Amplified PCR products from anthracene-growing isolates were purified using the “Ron's PCR-Purification” kit (BIORON GmbH, Germany). Each amplicon obtained was ligated in the “pGEM- T Easy” vector and then inserted into JM-109 competent cells (Promega, USA). Recombinant transformants were selected through blue and white colony screening and their plasmid DNA was extracted by the “GF-1 Plasmid DNA extraction” kit (Vivantis, Malaysia). Sequencing of the amplicons were carried out in Macrogen (South Korea) and Cemia (Greece) using vector primers SP6 (5'-TAT TTA GGT GAC ACT ATA G-3') and T7 (5'-TAA TAC GAC TCA CTA TAG GG-3') and deposited in GenBank under the accession numbers KM210226- KM210276.

Phylogenetic and statistical analysis

The 16S rRNA gene sequences of the bacterial strains isolated were assembled by using the “CAP3 Sequence Assembly Program” designated by Huang and Madan (1999). Sequence comparisons were accomplished by using the “BLAST” and “Seqmatch” platforms at NCBI and RDP (<http://www.ncbi.nlm.nih.gov/> & <https://rdp.cme.msu.edu/>, respectively). All closest matches were included in further phylogenetic analysis and aligned using the “ClustalW” bioinformatic tool (<http://www.genome.jp/tools/clustalw/>). Phylogenetic tree construction was carried out by Mega (Tamura et al., 2013) through calculation of the evolutionary distances with the method of Jukes and Cantor (1969). Tree topology was inferred by the “neighbor-joining” approach (Saitou and Nei, 1987) based on bootstrap analysis of 1000 trees.

Analysis of variance (ANOVA) based on Duncan's multiple comparison tests ($\alpha < 0.05$) was performed in order to estimate significance among treatment means. For the degradation efficiency data, percentage values were arcsine-transformed. Standard deviations ($n = 3$) were determined for the average concentrations obtained at the end of biological treatment with the isolated bacterial strains.

Results and discussion

The anthracene-degrading bacteria were isolated from an intermittently aerated and fed pilot-scale bioreactor treating municipal wastewater and operating under alternating nitrification-denitrification conditions (Table 1). The denitrification system was highly loaded, feeding under an F/M ratio of 0.468 kg BOD₅ kg⁻¹ VSS d⁻¹ (Table 1). However, bioreactor performance was efficient, showing good effluent characteristics and sludge settleability (Table 1), due to the alternating aerobic-anoxic conditions established (Melidis et al., 2014).

Total bacterial population grown on PCA plates was estimated in the initial inoculum as equal to 63.8 ± 1.5 (10^6) cfu ml⁻¹. Bacterial population growing on anthracene-containing plates increased significantly ($\alpha < 0.05$) from 36.0 ± 7.0 (10^6) in the initial inoculum (mixed liquor) to 103.8 ± 14.1 (10^7) cfu ml⁻¹ at the end of the first enrichment, while the respective population decreased by one magnitude at the end of the second enrichment procedure and was equal to 112.3 ± 32.7 (10^6) cfu ml⁻¹. The population increase in the first enrichment step may due to the addition of mixed liquor, which introduced exogenous carbon in the liquid culture and favored cometabolism phenomena. In parallel, anthracene accumulation in microbial cells was equal to $17.3 \pm 1.6\%$. At the end of each enrichment period, anthracene in the liquid medium was not detected.

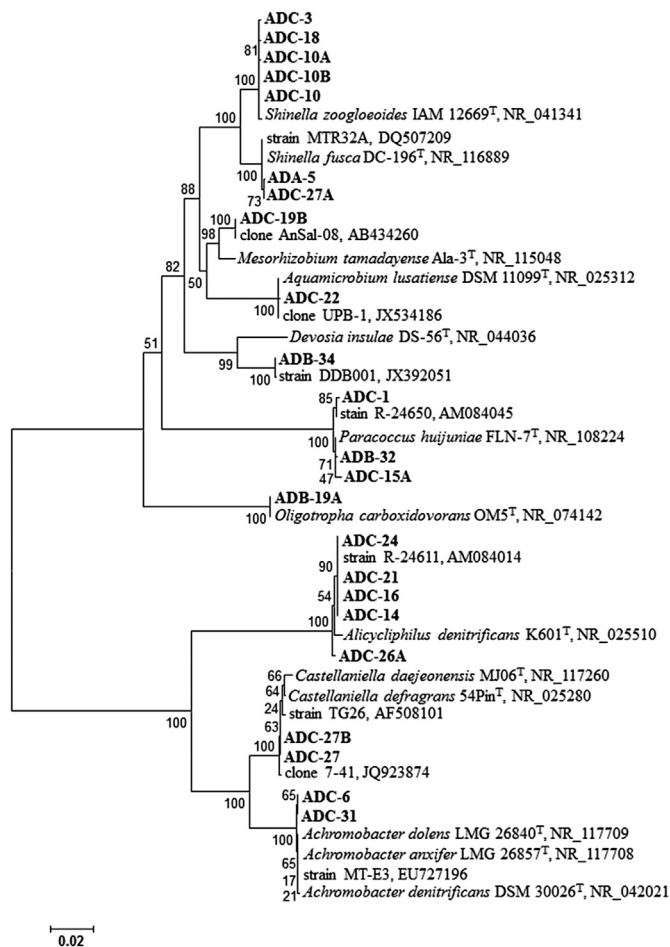


Fig. 1. Phylogenetic allocation of anthracene-degrading bacteria isolated from the activated sludge of the intermittently aerated and pulse fed bioreactor treating municipal wastewater within the Alphaproteobacteria and Betaproteobacteria.

A total of 51 bacterial strains were isolated from the activated sludge of the intermittently aerated and fed bioreactor using an anthracene-based growth medium. The isolates grown on anthracene-containing plates were further characterized by employing 16S rRNA gene sequence analysis and by examining their ability to degrade anthracene. Based on 16S rRNA gene sequencing, the isolated bacterial strains were classified into 17 operational taxonomic units (OTUs) (Table 2 and Figs. 1 and 2).

The largest OTU consisted of 9 isolates, which showed high phylogenetic similarities with the species *Pseudomonas citronellolis* (99.3–99.7%). They could decrease anthracene concentration, although in a relatively low degradation degree (Fig. 3).

Their closest phylogenetic relatives were associated with bacterial strains isolated from activated sludge or petroleum-contaminated soils, which were capable of degrading recalcitrant compounds, such as 3-chlorobenzoate and atrazine (Table 2). Members of OTU#2 (6 isolates) were phylogenetically related to *Microbacterium arabinogalactanolyticum* strains (99.7–100% similarity) with degrading ability on complex toxic compounds, i.e. *p*-nitrophenol and 4-aminopyridine (Table 2). In contrast to OTU#1 members, OTU#2 isolates resulted in approximately 70% anthracene reduction (Fig. 3). The isolates of OTU#3 highly decreased

anthracene concentration (by 61%) and were associated with *Shi-nella zoogloeoides* (ex *Zoogloea ramigera* strain) (99.8% similarity in 16S rRNA gene) (Table 2 and Fig. 3), a bacterium commonly found in organically polluted freshwaters and effluents (Unz, 1984). Interestingly, *Zoogloea* spp. appear to be favored in activated sludge systems operating under high organic loading rates (Adav et al., 2009). OTU#4 isolates (5 strains) were closely related to denitrifying *Alicyclophilus denitrificans* strains (99.7–100% similarity in 16S rRNA gene) isolated from activated sludge systems (Table 2) and could reduce anthracene concentration by 67% (Fig. 3). Members of OTU#5 (5 isolates) could decrease anthracene by 20% and were placed in the spectrum of the genus *Rhodococcus*, showing high phylogenetic similarities with the species *Rhodococcus aetherivorans* and *Rhodococcus ruber* (99.4–100%) (Table 2). OTU#6 strains (5 isolates) highly affiliated (99.9–100% similarity in 16S rRNA gene) with the strain *Stenotrophomonas acidaminiphila* LMG 19833 isolated from a denitrifying continuous upflow fixed-bed reactor (Mergaert et al., 2001), showing low anthracene reduction (Fig. 3). The three isolates of OTU#7 were related to *Paracoccus huijuniae* (99.5–99.9% similarity) (Table 2) and could reduce anthracene by a mean of 39% (Fig. 3). Four OTUs (OTUs#8, 9, 10 and 11) consisted of two isolates and decreased anthracene by 10–16%

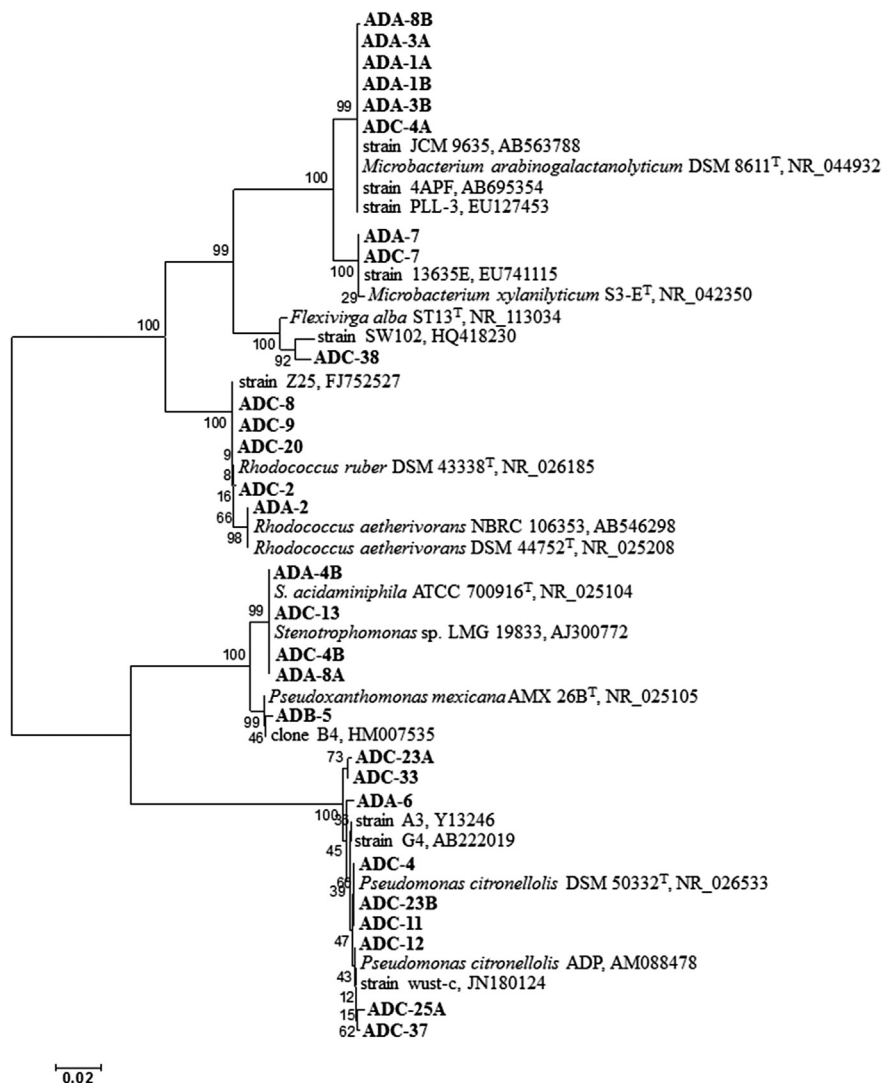


Fig. 2. Phylogenetic allocation of anthracene-degrading bacteria isolated from the activated sludge of the intermittently aerated and pulse fed bioreactor treating municipal wastewater within the *Gammaproteobacteria* and *Actinobacteria*.

(Table 2 and Fig. 3). OTU#8 isolates were phylogenetically related to the species *Castellaniella daejeonensis* and *Castellaniella defragrans* (98.6% and 98.4% similarity, respectively) (Table 2), with the denitrifying strain TG26, which was capable of degrading phenol, being their closest cultured relative (Baek et al., 2003). Members of OTUs#9 and #10 were other representatives of the genera *Microbacterium* and *Shinella*, showing phylogenetic similarities of 98.4% and 99.7–99.8% with the species *Microbacterium xylanilyticum* and *Shinella fusca*, respectively (Table 2). OTU#11 strains showed equal phylogenetic distances from the species *Achromobacter dolens*, *Achromobacter anxifer* and *A. denitrificans* (Fig. 1), although their closest relative was strain MT-E3 isolated from an industrial wastewater (Toups et al., 2010). The remaining OTUs (OTUs#12 to #17) comprised of a single isolate and were phylogenetically related to the type strains of the species *Pseudoxanthomonas mexicana* (99.8% similarity), *Aquamicrobium lusatiense* (99.9%), *Devosia insulae* (96.0%), *Mesorhizobium tamadayense* (98.7%), *Oligotropha carboxidovorans* (100%) and *Flexivirga alba* (98.3%). Their closest relatives have been previously isolated from wastewater and soil environments (Table 2). However, the strains belonging to OTUs represented by a single isolate, with the exception of the only *P. mexicana* isolate that slightly degraded anthracene, could not decrease PAH concentration (Fig. 3). However, isolate ADB-34 was distantly related to *Devosia insulae* (showing only 96% similarity in the 16S rRNA gene) (Table 2), representing therefore a novel phylogenetic linkage within the genus *Devosia*.

According to anthracene degradation data (Fig. 3), the greatest anthracene concentration reduction was observed for members of OTUs#2–4 (*M. arabinogalactanolyticum*, *Shinella zoogloeoides* and *Alicyclophilus denitrificans*-related strains) ($a < 0.05$), followed by those of OTUs#7, 5 and 10 (*Paracoccus huijuniae*, *Rhodococcus aetherivorans*/R. *ruber* and *Shinella fusca*-related strains). It appears that bacterial strains with some degree of anthracene biodegradability formed OTUs of high representativity (≥ 2 strains), while OTUs consisting of a single representative did not possess the ability to degrade anthracene. The latter strains appear to tolerate the selection pressure of anthracene rather being anthracene degraders.

Comparative evaluation of the anthracene degraders identified in the present study with anthracene-degrading bacteria reported in the literature revealed an intermediate anthracene degrading ability for the most effective anthracene degraders isolated (Table 3). Considering that the known anthracene-degrading bacteria have been mainly isolated from highly polluted sites, anthracene degraders from activated sludge, which have not been exposed to severe PAH-induced selection pressure, appear to exhibit a good degradation potential.

No *Alicyclophilus denitrificans* and *Shinella zoogloeoides* strains capable of degrading anthracene have been reported since now (Table 3). In addition, no known *Castellaniella* spp. capable of degrading PAH compounds have been identified. Indeed, this is the first report on PAH-degraders belonging to the genera *Alicyclophilus*,

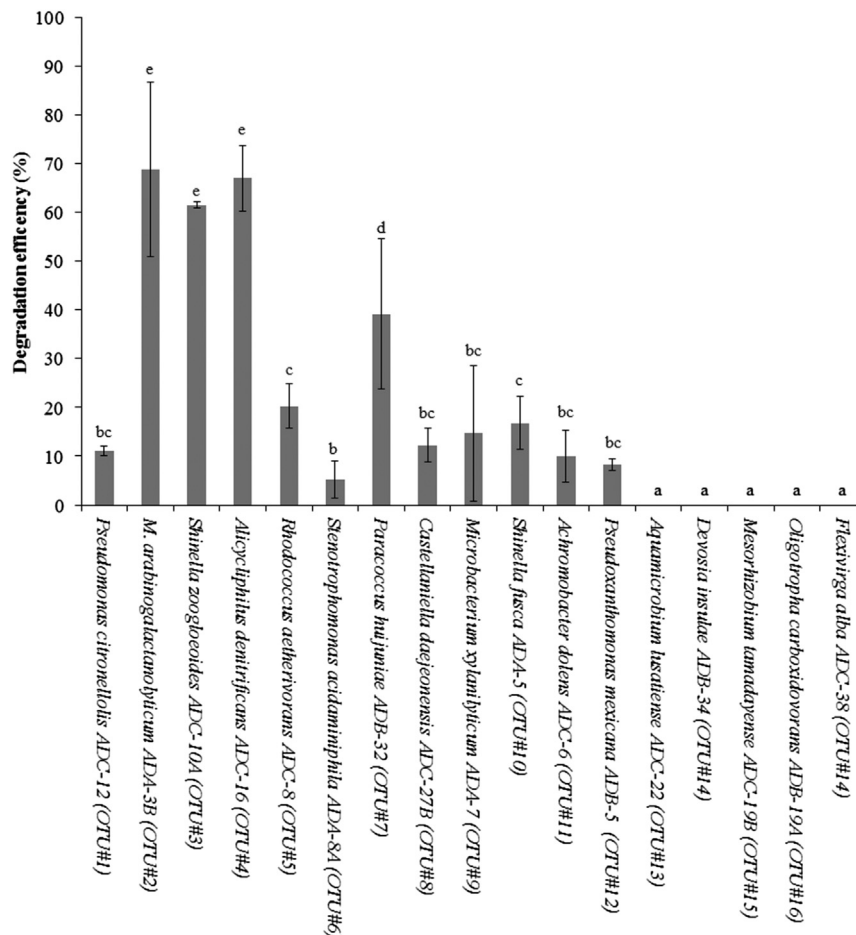


Fig. 3. Degradation efficiencies of anthracene-growing strains isolated from the activated sludge of the intermittently aerated and pulse fed bioreactor treating municipal wastewater. Statistically significant differences for $a < 0.05$ are denoted by lack of letters in common ($n = 3$).

Table 3

Phylogenetic allocation, degradation efficiency and isolation source of anthracene-degrading bacteria reported in the literature and in the present study.

Bacterial isolate	Anthracene concentration	Anthracene degradation (%)	Degradation period	Isolation source	Reference
<i>Pseudomonas citronellolis</i> 222A, 312A & 332C	250 mg L ⁻¹	56.5, 71.1 & 24.4	48 days	Petrochemical sludge	Jacques et al. (2005)
<i>Microbacterium</i> sp. SL10	50 mg L ⁻¹	90.1	21 days	Soils from oil-rich Niger Delta	Salam et al. (2014)
<i>Sphingopyxis</i> sp. PHPY	100 mg L ⁻¹	30	15 days	Oil-contaminated seawater	Pinyakong et al. (2012)
<i>Mycobacterium</i> sp. LP1	50 mg L ⁻¹	21	14 days	Agricultural topsoil	Pizzul et al. (2007)
<i>Sphingomonas</i> sp. PheB4	10 mg L ⁻¹	100	3 days	Surface mangrove sediments	Zhong et al. (2007)
<i>Arthrobacter</i> sp. Sphe3	400 mg L ⁻¹	18	4 days	Creosote-polluted soil	Kallimanis et al. (2007)
<i>Rhodococcus erythropolis</i> BZ4	20 mg L ⁻¹	100	28 days	Petroleum contaminated alpine soil	Margesin et al. (2013)
<i>Marteella</i> sp. AD-3	25 mg L ⁻¹	94.6	6 days	Saline petroleum-contaminated soil	Cui et al. (2012)
<i>Mycobacterium</i> sp. LB501T	20 mg L ⁻¹	>80	5 days	Contaminated soil	Bastiaens et al. (2000)
<i>Ochrobactrum</i> sp. VA1	3 mg L ⁻¹	88	5 days	Marine water	Arulazhagan and Vasudevan (2011)
<i>Rhodococcus</i> sp.	3 mg L ⁻¹	53	1 day	Contaminated river sediment	Dean-Ross et al. (2001)
<i>Bacillus</i> sp. SBER	480 mg L ⁻¹	83.4	6 days	Roots of poplar trees	Bisht et al. (2014)
<i>Jamibacter anophelis</i> sp. JY11	500 mg L ⁻¹	82.1	5 days	Polluted soil from oil refinery	Zhang et al. (2009)
<i>Paracoccus denitrificans</i> sp. Ophe1	100 mg L ⁻¹	^a	NR	PAH-contaminated soils	Zhang et al. (2004)
<i>Pseudomonas citronellolis</i> strains	10 mg L ⁻¹	11.1	5 days	Activated sludge	This study
<i>Microbacterium arabinogalactanolyticum</i> strains	10 mg L ⁻¹	68.8	5 days	Activated sludge	This study
<i>Shinella zoogloeoides</i> strains	10 mg L ⁻¹	61.4	5 days	Activated sludge	This study
<i>Alicyclophilus denitrificans</i> strains	10 mg L ⁻¹	66.9	5 days	Activated sludge	This study
<i>Rhodococcus aetherivorans/R. ruber</i> strains	10 mg L ⁻¹	20.2	5 days	Activated sludge	This study
<i>Stenotrophomonas acidaminiphila</i> strains	10 mg L ⁻¹	5.2	5 days	Activated sludge	This study
<i>Paracoccus huijuniae</i> strains	10 mg L ⁻¹	39.1	5 days	Activated sludge	This study
<i>Castellaniella daejeonensis</i> strains	10 mg L ⁻¹	14.8	5 days	Activated sludge	This study
<i>Microbacterium xylanilyticum</i>	10 mg L ⁻¹	16.8	5 days	Activated sludge	This study
<i>Achromobacter dolens/A. anxifer/A. denitrificans</i> strains	10 mg L ⁻¹	10.0	5 days	Activated sludge	This study
<i>Pseudoxanthomonas mexicana</i> strain	10 mg L ⁻¹	8.2	5 days	Activated sludge	This study

^a 0.5 mg day⁻¹ degradation rate. NR: not reported.

Castellaniella and *Shinella*. Furthermore, the isolate ADB-5 of the present study was the first reported *Pseudoxanthomonas mexicana* strain with PAH degradation ability. The *Paracoccus huijuniae*-related strains (members of OTU#7) identified also resulted in effective anthracene degradation, although this taxon has not been previously reported to decrease anthracene concentration. Besides, no *Microbacterium arabinogalactanolyticum*- and *Microbacterium xylanilyticum*-related bacterial strains with PAH degradability have been previously identified. These findings indicate that a new PAH-degrading bacterial diversity was identified in the denitrifying activated sludge of the intermittently aerated and fed bioreactor.

Despite the fact that several attempts have been performed to determine the efficiency of WWTPs (Wastewater Treatment Plants) to remove polycyclic aromatic hydrocarbons (including anthracene) from municipal wastewaters (Zhai et al., 2011), the composition of activated sludge biota capable of degrading PAHs still remains unexplored. Most of the PAHs-degrading bacteria have been isolated from petrochemical sludge and PAH-contaminated soils. Indeed, only a limited number of strains isolated from activated sludge treating municipal wastewater have been examined in terms of their ability to degrade PAHs species (Ma et al., 2005; Avramova et al., 2008). In the present study, a broad anthracene-degrading bacterial community was identified, indicating the multifunctionality and versatility of activated sludge treating municipal wastewater to cope with a wide variety of macro- and micro-pollutants.

In contrast to the general predominance of *Betaproteobacteria* in denitrifying activated sludge systems treating municipal wastewaters (Srinandan et al., 2011), the 46 anthracene-degrading isolates under study were mainly assigned to *Gamma*proteobacteria (30.4%), *Actinobacteria* (28.3%) and *Alphaproteobacteria* (21.7%), whereas *Betaproteobacteria* accounted only for 19.6%. Apparently, these biological agents comprised a specific part of activated sludge microbiota, which specialized in the degradation of the small proportion of such recalcitrant compounds present in the municipal wastewater. Moreover, as a consequence of the alternating

nitrification-denitrification mode of operation, several anthracene-degrading strains identified in the present study were phylogenetically related to known denitrifying bacteria (Table 2).

Conclusions

A broad diversity of anthracene-degrading bacteria was identified in the activated sludge of an intermittently aerated and fed bioreactor, indicating the multifunctionality and versatility of activated sludge treating municipal wastewater to cope with a wide variety of micro-pollutants. In addition, certain bacterial strains, which were related to the species *Alicyclophilus denitrificans*, *Microbacterium arabinogalactanolyticum* and *Shinella zoogloeoides*, can be considered as effective anthracene degraders, showing degradation efficiencies within 60–70%.

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