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Anaerobic degradation of benzene by enriched consortia with humic acids as terminal electron acceptors

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ABSTRACT

The anaerobic degradation of benzene coupled to the reduction of humic acids (HA) was demonstrated in two enriched consortia. Both inocula were able to oxidize benzene under strict anaerobic conditions when the humic model compound, anthraquinone-2,6-disulfonate (AQDS), was supplied as terminal electron acceptor. An enrichment culture originated from a contaminated soil was also able to oxidize benzene linked to the reduction of highly purified soil humic acids (HPSHA). In HPSHA-amended cultures, 9.3 μM of benzene were degraded, which corresponds to 279 ± 27 micro-electron equivalents (μEq) L^{-1}, linked to the reduction of 619 ± 81 μEq L^{-1} of HPSHA. Neither anaerobic benzene oxidation nor reduction of HPSHA occurred in sterilized controls. Anaerobic benzene oxidation did not occur in soil incubations lacking HPSHA. Furthermore, negligible reduction of HPSHA occurred in the absence of benzene. The enrichment culture derived from this soil was dominated by two γ-Proteobacteria phylotypes. A benzene-degrading AQDS-reducing enrichment originated from a sediment sample showed the prevalence of different species from classes β-, δ- and γ-Proteobacteria. The present study provides clear quantitative demonstration of anaerobic degradation of benzene coupled to the reduction of HA.

1. Introduction

Benzene is a widespread contaminant commonly found in aquifers due to leaks in underground fuel storage tanks, improper disposal techniques and spills of petroleum products. Contamination of water reservoirs by benzene has deserved particular scrutiny, as it is the most water-soluble and carcinogenic of all gasoline hydrocarbons [1].

Microbial degradation of benzene readily occurs under aerobic conditions by a wide variety of aerobic bacteria [2]. However, benzene often persists as contaminant in sedimentary environments because anaerobic conditions prevail [3–5]. Even though benzene is considered one of the most recalcitrant compounds in anaerobic environments, evidence collected during the last two decades indicates that anaerobic benzene degradation can be achieved under anaerobic conditions. Anaerobic benzene degradation has been demonstrated under methanogenic conditions [6,7] and linked to the reduction of different terminal electron acceptors (TEA), including sulfate [8–10], nitrate [7,11–13], Fe(III) [14–16], and Mn(IV) [17,18]. More recently, anaerobic benzene oxidation was demonstrated with graphite electrodes as a TEA in sediment incubations [19].

In the present study, humic substances (HS) are evaluated as TEA for benzene biodegradation. HS constitute the most abundant organic fraction of the biosphere accumulating in terrestrial and aquatic environments. Although these compounds were previously considered to be very inert, evidence has been accumulated indicating that they have active roles in the anaerobic oxidation of several distinct organic compounds by serving as TEA for humic-reducing microorganisms [20]. The redox mediating properties of HS have mainly been attributed to quinone moieties [21], which are very abundant in HS and the humic model compound, anthraquinone-2,6-disulfonate (AQDS), has been used in several studies documenting the role of HS as TEA during the anaerobic oxidation of priority pollutants, such as vinyl chloride, dichloroethene [22], phenol, *p*-cresol [23,24] and toluene [25]. Furthermore, HS have been shown to promote the anaerobic degradation of benzene by serving as an electron shuttle between Fe(III)-reducing bacteria and insoluble Fe(III) oxides [26]. Nevertheless, no direct

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evidence has been reported quantifying the role of HS as TEA linked to the anaerobic degradation of benzene. The aim of the present study was to evaluate the capacity of humic-reducing consortia to oxidize benzene with AQDS and humic acids (HA) as TEA. Benzene-degrading consortia were enriched under AQDS-reducing conditions and were phylogenetically characterized.

2. Materials and methods

2.1. Reagents, materials and inocula

Benzene (ACS grade, 99.8% purity) and AQDS were purchased from Sigma–Aldrich (Milwaukee, Mis.). Highly purified soil humic acids (HPSHA) were obtained from the International Humic Substances Society (IHSS, catalogue number 1S104H-5). All other reagents were of ACS grade. Two different inocula were selected for the present study based on their capacity to oxidize organic substrates (glucose, acetate) with AQDS as TEA during a preliminary screening and due to their past exposure to hydrocarbons. A soil sample derived from a petroleum refinery in Poza Rica (Veracruz, Mexico) was obtained from a place in which a petroleum spill occurred and it is referred to as PR soil in the present study. The sediment derived from Marland Lagoon in Ébano (San Luis Potosí, Mexico) has historically been exposed to hydrocarbons as the first petroleum production well exploited in Mexico was located next to the lagoon. Nowadays, periodic petroleum emanations naturally occur in the lagoon contaminating this water body. This consortium is referred to as ML sediment in the present study.

2.2. Benzene-degrading enrichment cultures with AQDS as TEA

Incubations were conducted in 117 mL glass serum bottles with basal medium, which composition is as follows ($g\,L^{-1}$): $NaHCO_3$, (1.68); NH_4Cl, (0.3); KH_2PO_4, (0.2); $MgCl_2{\cdot}6H_2O$, (0.03); $CaCl_2$, (0.1); Na_2S, (0.1) and $1\,mL\,L^{-1}$ of trace elements solution with the composition previously described [23]. AQDS (5 mM) was included in the medium as TEA and the pH was controlled at 6.7 ± 0.2 by the bicarbonate added and a headspace composed of N_2/CO_2 (80%/20%). Portions (50 mL) of basal medium were dispensed in serum bottles under anaerobic conditions (anaerobic hood with an atmosphere composed of N_2/H_2 (95%/5%)) and then inoculation took place by adding 10 g (dry weight) per liter of previously homogenized inocula. Bottles were sealed with Viton Stoppers (Maag Technic AG, Dübendorf, Switzerland) and aluminum crimps and were flushed with N_2/CO_2 (80%/20%) to establish anaerobic conditions. Benzene was supplied from an anaerobic stock solution to the initial concentration of 50 μM referred to the liquid volume. After benzene biodegradation was observed linked to AQDS reduction, consecutive cycles were performed by replacing the medium in an anaerobic hood (atmosphere composed of N_2/H_2 (95%/5%)) by freshly prepared anaerobic medium. Strict anaerobic conditions were guaranteed by flushing the basal medium with N_2/CO_2 (80%/20%), by adding sulfide as a reducing agent ($0.1\,g\,L^{-1}$) and by allowing initial AQDS reduction (up to 0.1 mM with endogenous substrates remaining from previous cycles) before addition of benzene under strict anaerobic conditions. Benzene degradation and AQDS reduction was determined as described below. Sterile controls were prepared under the same experimental conditions and autoclaved for 20 min at 120 °C two times before addition of benzene. Incubation controls lacking benzene were also included in order to correct for the endogenous reduction of AQDS. Finally, biologically active controls incubated without AQDS were also included to verify the association between anaerobic benzene oxidation and AQDS reduction. All experimental treatments were performed by triplicate and statically incubated in the dark at 28 °C. Manual shaking during sampling was conducted to promote homogenous distribution of benzene.

2.3. Anaerobic benzene oxidation with humic acids as TEA

Enriched consortia in which benzene biodegradation linked to AQDS reduction was observed were evaluated for their capacity to achieve anaerobic benzene oxidation with HPSHA as TEA. Microbial incubations were performed in 60 mL glass serum bottles with basal medium (25 mL) provided with $5\,g\,L^{-1}$ of HPSHA. Benzene was supplied from an anaerobic stock solution at the initial concentration of 30 μM referred to the liquid volume. Previous studies documented the role of this HA source as TEA during the anaerobic mineralization of toluene by enriched sediments [25]. The electron accepting capacity of this humic sample was determined as described below. During benzene biodegradation, benzene concentration and reduction of HPSHA were monitored as described below. Sterile controls were prepared under the same experimental conditions and autoclaved for 20 min at 120 °C two times before addition of benzene. Incubation controls lacking benzene were also included in order to correct for the endogenous reduction of HPSHA. Finally, biologically active controls incubated without HPSHA were also included to verify the association between anaerobic benzene oxidation and the reduction of HPSHA. All experimental treatments were performed by triplicate and statically incubated in the dark at 28 °C. Manual shaking during sampling was conducted to promote homogenous distribution of benzene.

2.4. DNA extraction and PCR amplification

Enriched biomass samples derived from PR soil and ML sediment were washed with 1 mL ice-cold PBS 1: 2-propanone (10:1 vol/vol) and then centrifuged for 15 min at 14,000 rpm. A second wash was performed with PBS 1: ethanol (1:1 vol/vol) and a third one with PBS 1. Genomic DNA was extracted by enzymatic lysis and chloroform-isoamyl (24:1) alcohol extraction and 1 volume isopropanol precipitation based on a previously published protocol [27]. The yield and quality of DNA were analysed electrophoretically on 1% (wt/vol) agarose gels.

Bacterial 16S rRNA gene amplifications were performed using 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-TACGGYTACCTTGTTACGACTT-3′) primers and conditions were modified from Bond et al. [28] (annealing 57 °C; 30 cycles). Reaction mixture consisted in primers (0.2 μM each), 200 μM dNTPs, 2 mM $MgCl_2$, 1× PCR buffer and 0.04 U/μL *GoTaq* DNA Polymerase (Promega). Amplifications were performed using a *DNA Engine Peltier Thermal Cycler* (Bio-Rad).

2.5. Cloning of 16S rRNA

Four clone libraries were constructed: Original PR soil and ML sediment samples, and enriched PR soil and ML sediment samples obtained under AQDS-reducing conditions with benzene as a sole energy source. Amplicons were ligated into *pGEM-T Easy* cloning vector (Promega) and transformed by thermal shock of competent *Escherichia coli* TOP-10F' cells. Plasmidic DNA was extracted from all transformed colonies by the method of Birnboim and Doly [29]. A terminal restriction fragment length polymorphism analysis (T-RFLP) was performed in order to avoid redundancies; plasmidic DNA was digested in a single reaction with *EcoRI* and *MspI* [30]. Enzymatic digestion was performed by incubating 3 μL of the insert with 0.3 U of each enzyme and the corresponding enzyme buffer at 37 °C for 4 h. The digestion products were analysed in 2%

agarose gels in TAE 1 and stained on 0.5 μg mL^{-1} ethidium bromide. Colonies showing distinct fingerprint patterns were sequenced using the vector-specific M13f and M13r primers in a DNA automated sequencer *ABI Prism Model 377r* (Applied Biosystems, USA).

2.6. Phylogenetic analysis

Electropherograms were transformed into contiguous fasta sequences with *DNA baser* software (www.dnabaser.com). Sequences orientation and chimeras were detected by using *Orientation Checker* v.1.0 and *Pintail* v. 1.0 [31,32]. The redundant phylotypes (sharing ≥97%) were collapsed in operational taxonomic units (OTUs) by distance analyses using the *Ribosomal Database Project* (RDP) web page (http://rdp.cme.msu.edu) [33]. The 16S rRNA gene sequences were aligned with *ClustalW* algorithm and optimized manually with type sequences using *eBioX* v. 1.5.1. [34]. Alignments included the phylotypes and the closest sister genera reported in *The All-Species Living Tree* project [35] and the Bergey's Manual [36]. Posterior probability and topology of the phylogenetic trees were inferred by Bayesian analysis with *Mr. Bayes* v. 3.1.2 [37] defining the parameters GTR + I + G, after a consensus of 3×10^5 generations ("burnin" of 50% a standard deviation of 0.003). The 16S rRNA gene sequences were deposited in the GenBank database under accession numbers: HQ602825–HQ602872 and HQ694510–HQ694512.

2.7. Analytical methods

Benzene concentrations were determined in 100 μL headspace samples by gas chromatography (Agilent Technology, Model 6890N) and a flame ionization detector. The chromatograph was equipped with a capillary column (Agilent DB-624) and nitrogen (25 ml per min) was used as a carrier gas. The temperature of the injection port, oven and detector, were 230, 60 and 230 °C, respectively. Standard bottles were previously autoclaved for 20 min at 120 °C two times and incubated at 28 °C overnight before adding benzene.

Electron accepting capacity (EAC) of HPSHA was determined by the ferrozine technique in a N_2/H_2 (95%/5%) anoxic chamber following the protocol described by Lovley et al. [38]. The biological method to determine the EAC of this humic sample was based on the reduction by *Geobacter sulfurreducens* according to the protocol of Lovely et al. [38]. Reduction of AQDS (as AH_2QDS) was determined in the same anaerobic chamber according to Cervantes et al. [39]. Reduction of HPSHA was determined by the ferrozine technique according to Lovley et al. [38]. Subsamples in which Fe(III) citrate was no added were also monitored by the ferrozine technique in order to subtract the reducing equivalents received by intrinsic Fe(III) present in HPSHA.

3. Results

3.1. Anaerobic degradation of benzene with AQDS as TEA

ML sediment degraded benzene under anaerobic conditions when AQDS was provided as TEA (supplementary data, Fig. S1). After more than two months of lag phase, concomitant benzene degradation and AQDS reduction occurred in biologically active sediment incubations. No benzene degradation was observed in sediment incubations lacking AQDS or in sterilized controls including AQDS. About 5% of the benzene initially spiked disappeared in all experimental treatments during the first 4 weeks of incubations presumably due to adsorption on the sediment (no benzene removal occurred in sterilized controls without sediment); however, no further benzene removal was detected in sterile controls and in sediment incubations lacking AQDS after this period. After 112 days of incubation, 12.9 ± 2.3 μM of benzene (corrected for the amount adsorbed) were degraded linked to the reduction of 293 ± 21 μM of AQDS (corrected for the endogenous control incubated in the absence of benzene). Thus, the stoichiometric relationship established during the anaerobic biodegradation of benzene was 1 μmol benzene degraded per 22.7 μmol of AH_2QDS produced, which is higher than the expected from the following stoichiometry:

$$C_6H_6 + 15\,AQDS + 12\,H_2O \rightarrow 6\,CO_2 + 15\,AH_2QDS$$

Nevertheless, the results suggest that the amount of benzene degraded was completely oxidized by ML sediment. The higher than expected ratio benzene degraded/AQDS reduced may partly be explained by the biodegradation of adsorbed benzene, which could not be quantified.

Anaerobic benzene oxidation was also observed in PR soil incubations (supplementary data, Fig. S2). Adsorption of benzene (~45% of the amount initially added) occurred in all experimental treatments during the first 3 months of incubation. However, after this period of time no further benzene removal was observed in sterilized controls. Clear distinction between abiotic benzene removal (*e.g.* adsorption) and biodegradation could be established after 4 months of incubation since benzene degradation only occurred in biologically active PR soil incubations. In the absence of AQDS, 2.67 ± 0.17 μM of benzene were degraded after 198 days of incubation. No methanogenic activity was detected during the anaerobic degradation of benzene in PR soil incubations lacking AQDS, suggesting that unidentified TEA present in this consortium were responsible for the anaerobic benzene oxidation observed. Moreover, simultaneous benzene biodegradation and AQDS reduction occurred in AQDS-amended soil incubations. After 198 days of incubation, 3.4 ± 0.67 μM of benzene were degraded (corrected for the amount of benzene degraded in the absence of AQDS) and 344 ± 20 μM of AH_2QDS were produced (corrected for the endogenous control lacking benzene), which corresponds to a ratio of 1 μM of benzene degraded per 101 μM of AH_2QDS produced. The ratio benzene degraded: AQDS reduced is very distant from that expected from the stoichiometry (see above). Thus, it is conceivable to assume that an important amount of benzene adsorbed might have been oxidized in PR soil incubations amended with AQDS. In fact, considering the amount of AH_2QDS produced and the stoichiometry, the expected amount of benzene that might have been oxidized under these conditions is ~23 μM, corresponding to 46% of benzene originally spiked, which agrees with the amount of benzene adsorbed.

Both benzene-degrading quinone-reducing consortia were incubated in several consecutive cycles in which AQDS reduction was consistently observed with benzene as a sole energy source for 18 months (data not shown). Enriched consortium derived from PR soil was selected for documenting the anaerobic benzene oxidation with HPSHA as TEA based on its superior capacity to achieve anaerobic benzene oxidation with AQDS as TEA (see below).

3.2. Anaerobic oxidation of benzene with humic acids as TEA

The EAC of HPSHA was measured in order to supply with enough EAC to achieve complete anaerobic benzene oxidation (30 μM). Chemical EAC determined in a H_2/Pd reaction system yielded 342 ± 23 micro-reducing equivalents (μEq) g^{-1}. Moreover, biological EAC determined with *G. sulfurreducens* yielded 216 ± 6 μEq g^{-1}. Thus, 5 g HPSHA L^{-1} was supplied in order to have an excess of EAC for anaerobic benzene oxidation (*e.g.* 30 μM of benzene require 900 μEq L^{-1} for complete oxidation).

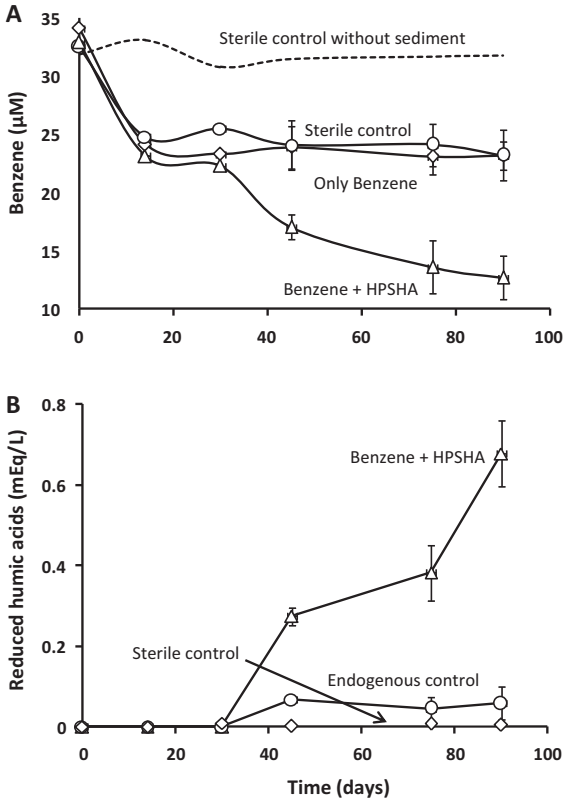

Fig. 1. Anaerobic benzene degradation (A) coupled to reduction of HPSHA (B) by PR soil in incubations containing bicarbonate-buffered medium supplemented with 5 g of HPSHA per liter. Endogenous controls were incubated in the absence of benzene. Sterile controls including HPSHA. Data represent average from triplicate measurements and standard deviations in each treatment.

An enrichment culture derived from PR soil achieved anaerobic benzene oxidation linked to the microbial reduction of HPSHA (Fig. 1). Adsorption to PR soil accounted for ~40% of benzene removal, which occurred during the first 4 weeks of incubation in all experimental treatments including PR soil, but no benzene removal was observed in chemical controls lacking PR soil (Fig. 1). However, a clear distinction between adsorption and biodegradation could be established after this incubation period. Certainly, anaerobic benzene oxidation was only observed in soil incubations amended with HPSHA after this period of time. Furthermore, the role of HPSHA as TEA supporting anaerobic benzene oxidation could be corroborated by measuring a significant amount of reduced humic acids as compared with the endogenous control lacking benzene (Fig. 1). In fact, $9.3 \pm 0.4\,\mu M$ (corrected for the sterilized control) of benzene were degraded in HPSHA-amended cultures, which corresponds to $279 \pm 27\,\mu Eq\,L^{-1}$, linked to the reduction of $619 \pm 81\,\mu Eq\,L^{-1}$ of HPSHA (corrected for the endogenous control lacking benzene). Neither anaerobic benzene oxidation nor reduction of HPSHA occurred in sterilized controls. Furthermore, benzene oxidation did not occur in soil incubations lacking HPSHA. The superior reduction of HPSHA as compared with that expected from the anaerobic benzene oxidation quantified could be explained by biodegradation of adsorbed benzene, which could not be quantified.

3.3. Phylogenetic characterization of benzene-degrading humus-reducing enriched consortia

The characterization of the four clone libraries: original PR soil and ML sediment and AQDS-benzene enriched PR soil and ML sediment; resulted in a total of 227 clones which were grouped into 32 different digestion patterns by T-RFLP analysis and 72 clones were sequenced redundantly to confirm the patterns. From the 72 sequenced clones, 23 sequences (30%) were identified as chimeras and were excluded from the phylogenetic analysis.

Tree branching was resolved unambiguously for most *16S rRNA* gene OTUs at level of genera. However, five clones (C11, A6, A10, E1 and E2) remained affiliated until Class level. A posterior probability of 100% was calculated in most nodes. In some cases, the weakly supported nodes were examined with sequences from clones available at the GenBank database, and the branching within the family reached a clear definition. A phylogenetic tree based on the sequences derived from this study and GenBank accession numbers is shown in Fig. 2.

AQDS-benzene enrichment libraries were predominantly composed of members of the *phylum Proteobacteria* and included the highest proportion of OTUs (48%). In PR consortia, two OTUs (C9 and C3) were not observed in the original sample and were assigned to γ-Proteobacteria. OTU C9 was closely related to *Pseudoxanthomonas* clade with a posterior probability of 100%. OTU C3 (HQ602844) allowed a phylogenetic assignment to γ-Proteobacteria in *Pseudomonas* clade. As for OTU C3 the search against the GenBank and RDP databases (using BLAST and Classifier tools, respectively), rendered the following results: BLAST retrieved sequences where the best hit showed a similarity of 93% to *Pseudomonas* clone (EU266810) and Classifier assigned a similarity of 94% to *Pseudomonas* genera. However, caution for species identification by local alignment (BLAST and Classifier) was taken into account for all phylotypes and the phylotype identification was preferably interpreted by phylogenetic analysis. This assignment revealed a close relation of phylotype C3 to the Pseudomonadaceae clade, because preliminary phylogenies discarded a clustering to the Moraxellaceae (the other family in Pseudomonadales), Halomonadales and Oceanospirillales clades. In ML consortia, 22 OTUs were detected and resolved at species level, the sequences were predominantly composed by γ-Proteobacteria species. Moreover, several species belonging to the following phyla were detected in ML sediment enrichment culture: *Bacteroidetes*, *Chloroflexi*, uncultured phylum TM-7 and as well as the classes β-, δ- and γ-Proteobacteria.

4. Discussion

The aim of the present study was to evaluate the capacity of humic-reducing consortia to achieve anaerobic benzene oxidation with HPSHA and the humic model compound, AQDS, as TEA. Multiple experimental proofs evidenced the role of humic substances as TEA during anaerobic benzene oxidation by enriched consortia. Both inocula studied (ML sediment and PR soil) could couple the anaerobic oxidation of benzene to AQDS reduction. Furthermore, a benzene-degrading enrichment culture derived from PR soil achieved anaerobic benzene oxidation when HPSHA were supplied as TEA. Concomitant anaerobic benzene oxidation and microbial reduction of HPSHA occurred in soil incubations supplemented with this TEA. Neither anaerobic benzene oxidation nor reduction of HPSHA occurred in sterilized controls. Furthermore, benzene oxidation did not occur in soil incubations lacking HPSHA. Furthermore, minor reduction of HPSHA occurred in endogenous controls lacking benzene. The present study shows a clear quantitative demonstration that anaerobic benzene oxidation can be accomplished by anaerobic consortia with humic acids as TEA.

Previously, it was hypothesized that HA had served as a direct electron acceptor during benzene biodegradation when HA were added as chelators to increase Fe(III) oxide bioavailability for a benzene-degrading Fe(III)-reducing consortium in contaminated

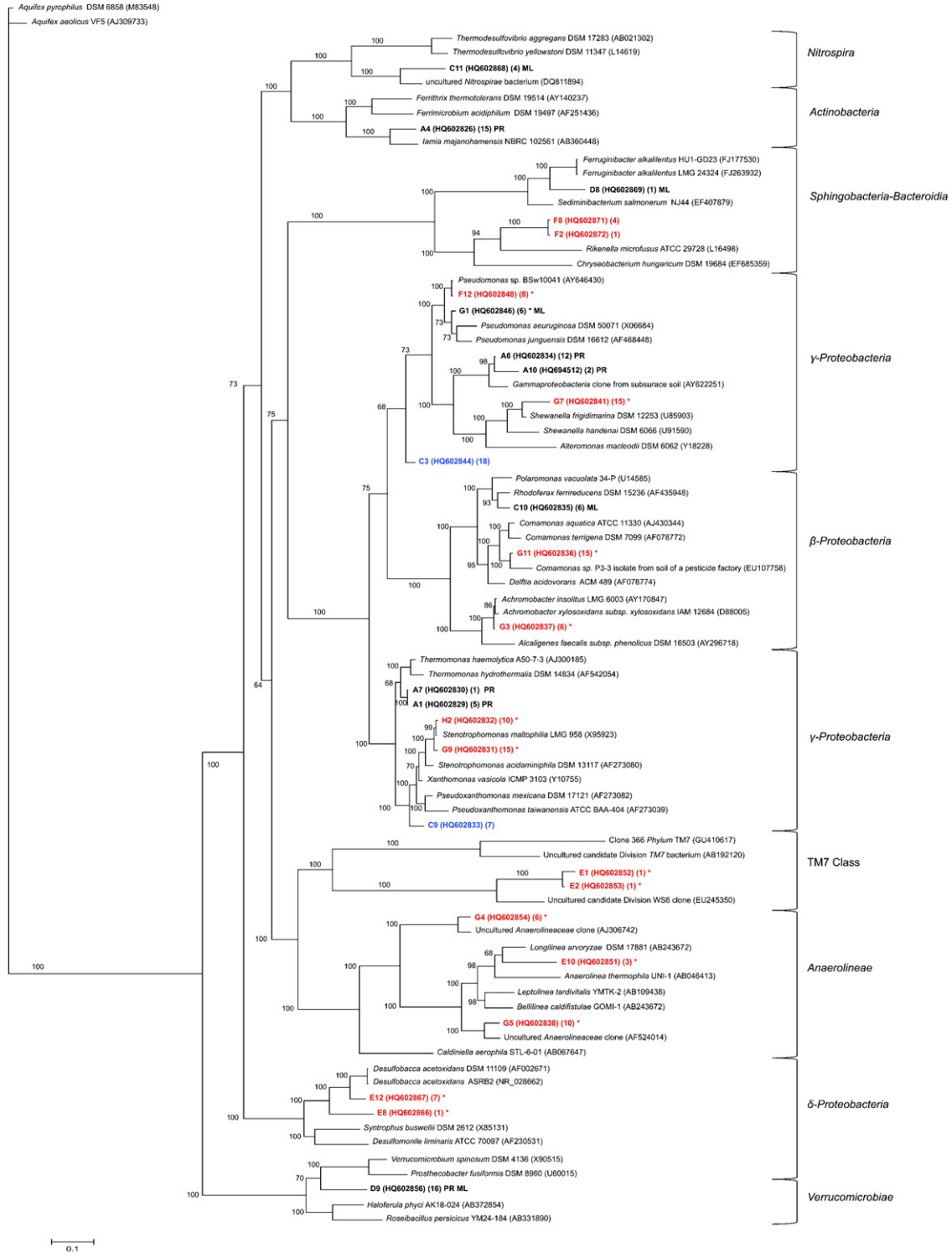

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences obtained from the clone libraries in this study. Two species of *Aquifex* were used as outgroup. The posterior probability support is indicated in nodes. The number of collapsed phylotypes assigned to OTU by sequencing and T-RFLP pattern analysis is indicated in parenthesis next to the GenBank accession number. Phlylotypes in black font correspond to members of original samples (ML sediment and PR soil origin site). The color font mark the phylotypes detected in the benzene-AQDS enrichments: red (ML sediment) and blue (PR soil). The symbol * denotes the genera or family reported to biodegrade aromatic hydrocarbons. The scale bar represents expected changes per site. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

sediment [38]. This hypothesis was based on the observation that HA promoted benzene biodegradation better than synthetic chelators (*e.g.* EDTA and NTA) even though humus had inferior chelating properties [26]. The mechanism proposed implies that benzene had been degraded with HA serving as TEA and the obtained reduced HA had been recycled back to the oxidized form by chemical reaction with Fe(III) oxides present in the cultures. However, the reduction of HA during benzene biodegradation was not demonstrated and the EAC of HA could not be differentiated from its impact as chelating agent. The impact of AQDS on the anaerobic benzene oxidation was also studied in three different sites of Fe(III)-reducing sediments [14]. Stimulation of anaerobic benzene oxidation was

observed at one site when 600 μM AQDS was applied, which may have been due to the use of AQDS as TEA, but the reduction of AQDS was not demonstrated. The same sediment sample did not oxidize benzene when 300 μM AQDS was applied, yet the amount of benzene added (12 μM) would have only required 180 μM AQDS for complete oxidation.

The role of HA and the humic model compound, AQDS, as TEA has previously been demonstrated during the anaerobic oxidation of other priority pollutants, such as vinyl chloride, dichloroethene [22], phenolic compounds [23,24], and toluene [25]. Thus, these findings suggest that HS may play a significant role during the intrinsic bioremediation of contaminates sites by serving as TEA. The ubiquity of HS and humic-reducing microorganisms in many different terrestrial and sedimentary environments [39,40] further emphasize their potential impact in the anaerobic oxidation of priority pollutants. Moreover, reduced HA can be recycled to its oxidized form by chemical reaction with metal oxides commonly found in anaerobic habitats [38,41], thus allowing its role as TEA at sub-stoichiometric concentrations [25]. However, a greater contribution of metal oxides on the recycling of HA is expected in environments that continuously shift from anoxic to aerobic conditions, such as shallow water bodies, so that metal oxides could be replenished. Reduced HA can also be recycled back to their oxidized form by transferring electrons to electron-accepting pollutants, such as azo dyes, nitroaromatics, polyhalogenated compounds and radionuclides [20]. Furthermore, reduced HA could also serve as electron donor for the microbial reduction of other electron acceptors, such as nitrate, nitrite, nitrous oxide, fumarate, perchlorate, arsenate and selenate [42–44].

The phylogenetic characterization of the microbial communities sampled from four PR soil and ML sediment clone libraries via 16S rRNA gene targeted T-RFLP analyses revealed that a significant OTU fraction was detected exclusively after enrichment with AQDS-benzene (22 of 32 OTUs). In ML sediment the selective AQDS-benzene enrichment revealed an occurrence of five OTUs belonging to the anaerobic taxa *Anaerolineaceae* and *Desulfobacca acetoxidans*, which have previously been reported to degrade monoaromatic hydrocarbons under different redox conditions [45,46]. Additionally, in ML sediment AQDS-benzene enrichment the facultative genera *Shewanella, Pseudomonas* and *Comamonas* were detected, which agrees with previous reports documenting the anaerobic degradation of benzene by other members of β-Proteobacteria, such as species from *Dechloromonas* [47,48] and *Pelomonas* [49]. On the other hand, in AQDS-benzene enriched PR soil a Pseudomonadaceae phylotype was observed, which is a relevant finding since members of this genera have also been reported to degrade monoaromatic compounds [2,50,51]. The strict aerobes *Achromobacter* and *Stenotrophomonas* have been reported to metabolize monoaromatic hydrocarbons [52]. The *phylum* TM-7 has no cultured species; however, it has been described in anoxic conditions where the biodegradation of nitrogen containing aromatic compounds occurs [53]. Furthermore, a wide variety of microorganisms belonging to several phyla identified in both enrichments, have previously been reported to reduce either HS or AQDS [54]. Therefore, the results suggest that the enriched microorganisms detected are involved during the anaerobic degradation of benzene using HA or AQDS as TEA.

5. Conclusions

The anaerobic degradation of benzene coupled to the reduction of HPSHA or AQDS was demonstrated in two enriched consortia. Both benzene-degrading enrichment cultures were phylogenetically characterized and the results indicate the prevalence of different species from classes β-, δ- and γ-Proteobacteria. The present study provides a clear quantitative demonstration of anaerobic degradation of benzene coupled to the reduction of HA.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2011.08.028.

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