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Epsilonproteobacteria dominate bacterial diversity at a natural tar seep

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ABSTRACT

The bacterial diversity of a naturally seeping bitumen source was investigated by *16S rRNA* gene cloning and sequencing. *Epsilonproteobacteria* were shown to dominate the bacterial diversity in the underground water and within the bitumen, representing ca. 75% of the total bacterial diversity. These *Epsilonproteobacteria* were dominated by *Sulfurimonas* OTUs, while *Sulfurovum* and *Arcobacter* OTUs completed the remaining diversity. *Epsilonproteobacteria* are sulfur-oxidizer, nitrate-reducing chemo-lithoautotrophic bacteria, unable to use most organics for growth but capable of CO_2 fixation. Thus, reduced sulfur species, but not the complex organic matter of the tar, are utilized for growth by bacterial communities at the Puy-de-la-Poix. The large prevalence of populations of *Epsilonproteobacteria* is a clear indication that crude oil offers a competitive ecological niche for these organisms.

Mots clés :
Epsilonprotéobactéries
Dégradation du pétrole
Cycle du soufre
Sulfurimonas
Source de bitume

RÉSUMÉ

Nous avons caractérisé la diversité bactérienne au niveau d'une source naturelle de bitume altéré par séquençage d'une librairie de gènes *ribosomiques 16S clonés*. Nous avons observé que la diversité bactérienne est largement dominée par des Epsilonprotéobactéries dans le bitume et dans l'eau souterraine. Ces Epsilonprotéobactéries représentent 75 % de la diversité bactérienne totale. Cette diversité est dominée avant tout par les OTU de *Sulfurimonas*, que complètent des OTU de *Sulfurovum* et *Arcobacter*. Les Epsilonprotéobactéries sont des bactéries chimiolithotrophes, oxydant les sulfures et réduisant les nitrates, incapables d'assimiler les molécules organiques, mais capables de fixer le carbone des carbonates dissouts. Ainsi, les composés soufrés, et non la matière organique complexe du bitume, seraient dégradés par les communautés bactériennes du Puy-de-la-Poix. La présence massive d'Epsilonprotéobactéries indique que l'interface eau hydrothermale/bitume offre une niche écologique favorable à ces microorganismes.

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1. Introduction

There is growing evidence that crude oil is degraded *in situ* by anaerobic prokaryotes. Heavy oils, tar and gas are the legacy of this microbial degradation over geological times. Petroleum reservoirs are important habitats within the deep biosphere, in which microbial consortia live at the oil–water interface, despite the drastic physicochemical conditions in the reservoirs, e.g., temperatures up to 190 °C in the Elgin–Franklin fields, North Sea [1], salinities up to 400 g/l in the Verkhnechona field, Russia [2], or the lack of oxygen. Many anaerobic prokaryotes have been retrieved both by molecular and cultural approaches from such ecosystems [3]. The microbial mineralization of OM relies on the cooperation of different groups of anaerobic microorganisms in sequence: fermenters, syntrophs, and methanogens [3]. This microflora is composed of genera belonging to the bacterial phyla *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* and methanogenic *archaea*, such as *Methanomicrobiales*, *Methanosarcinales*, and *Methanobacteriales* [4]. The bacterial diversity in oil reservoirs often presents large populations of *Epsilonproteobacteria* [5,6]. *Epsilonproteobacteria* are major sulfur-oxidizers, nitrate reducers autotrophs and mixotrophs, whose contribution to the sulfur cycle is essential in different environments, such as deep-sea hydrothermal vents and sediments [7], sulfidic cave springs [8], or episymbiotic associations [9]. In these environments, the diversity of *Epsilonproteobacteria* is often dominated by a single genus: *Sulfurovorum* in sulfidic biofilms, *Sulfurimonas* in the Colleville oil field, *Arcobacter* in the Pelican Lake oil field or *Sulfuricurvum* in the Athabasca oil sand reservoir. Although *Epsilonproteobacteria* are frequent, or dominate the bacterial diversity in different oil environments, it is unlikely that they partake in the breakdown of the organic matter of the oil, but more likely oxidize the fraction of sulfur compounds of the oil [5,6].

In the absence of the appropriate geologic structure to form a reservoir, crude oil and gas formed in the depth naturally seep out at the surface. These oils are often heavily degraded as a result of microbial activity before or during the migration to the surface, which raises questions about whether the oil-degrading prokaryotes migrate to the surface along with the oil, and whether petroleum degradation persists at the surface. To address this question, we investigated the microbial populations at the Puy-de-la-Poix, a small, naturally flowing seep of heavily degraded oil, or bitumen, in the Limagne region (France). We show here that as observed in other degraded-oil environments, the bacterial diversity at the Puy-de-la-Poix was characterized by a large majority of *Epsilonproteobacteria* of the *Sulfurimonas* genus.

2. Methods

Sampling: The "Puy-de-la-Poix" is a naturally flowing tar source located near the airport of Clermont-Ferrand (France, 45.7822 N 3.14642 E, 340 m, Fig. 1). The source appears as a small pool of ca. 3 m by 2 m, which may be covered by rain fall water according to the season, and overflows into the adjacent agricultural fields. At the time of sampling, the air temperature was 6 °C. The tar formed a thick mass on top of the source and the water covering the tar was ca. 2–5 cm deep. The water above the tar was sampled aseptically using sterile syringes (100 ml, surface water samples, SW). The remaining surface water was removed to avoid cross contamination with waters below the tar. Underground waters (UW, 100 ml) were sampled below the tar using sterile tubing punched through the tar. The chemical composition of the underground water can

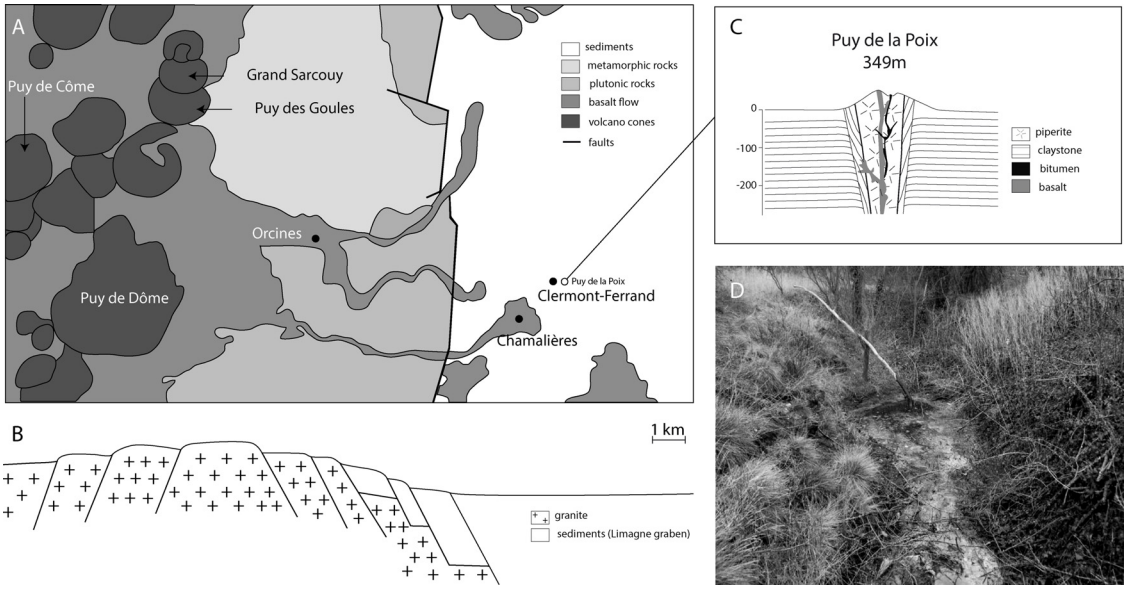

Fig. 1. Geographical and geological characteristics of the Puy-de-la-Poix. Panel A: Geologic map close up of the Clermont-Ferrand area. The Puy-de-la-Poix is located east of the city, next to the airport. Panel B: Corresponding cross section. Panel C: detailed geologic structure at the Puy-de-la-Poix Panel D: The seep flows from the top of the ancient volcano into the nearby agricultural fields.

be found in Table S1. A 3-cm-thick, 25 × 25 cm square piece of bitumen was cut aseptically with a sterile blade (bitumen sample, B).

16S library construction: Metagenomic DNA was prepared from 1 g of bitumen or 50 ml of water sample as described previously [10]. The complete *16S rRNA* gene was amplified using the universal eubacterial primers pA and pH [11] and cloned into pGEM-T easy (Stratagene, France) to generate the UW, B, and SW libraries. The complete sequence of the *16S* gene was determined by Sanger sequencing. GenBank accession numbers for representative groups of *Epsilonproteobacteria* are JX403021-JX403066.

Diversity analyses: Sequences were classified using the RDP classifier with a confidence threshold of 80% (https://www.rdp.cme.msu.edu). Mothur 1.36 [12] was used to eliminate chimera (*chimera.uchime*), assign sequences to OTUs (*align.seqs, dist.seqs, cluster*, OTUs, 97%), get representative sequences (*get.oturep*), and calculate diversity values ($H_{Shannon}$ and S_{Chao1}). Phylogenetic trees were reconstructed with Seaview using PhyML, and supported by 1000 boostrap repetitions [13].

Cultivation: Growth was assayed in media specific for *Epsilonproteobacteria*: *Thiomicrospira* medium DSM142, *Sulfurospirillum* medium DSM771 or *Sulfurimonas* medium DSM1053 (http://www.dmsz.de). Growth media were supplemented with electron donors (lactate, acetate, fumarate, formate, CO_2) and acceptors (SO_2, SO_4, NO_3, SeO_3, thiosulfate) at 5 mM final concentration. Twenty-milliliter enrichment cultures were set in sealed 50-ml serum vials under nitrogen atmosphere. Enrichment cultures were started with a 200-mg piece of bitumen. Cell counts were determined at least twice a week using a Thoma cell. Positive cultures were subcultured by serial dilution every two weeks until pure cultivable isolates were obtained. Purity was estimated by visual inspection of the culture and PCR amplification, cloning and sequencing of the *16S rRNA* gene.

3. Results and discussion

Bacterial diversity at the Puy-de-la-Poix. A total of 159 16S rDNA bacterial sequences, e.g., 58, 49 and 52 clones from the bitumen (B), underground water (UW) and surface water (SW) libraries, respectively, were obtained after quality and chimera screening. The sequences clustered into 48 different OTUs, belonging to six phyla (Table S2). The diversity is dominated by proteobacteria, e.g., 96,5%, 93,9%, and 84,6% in the B, UW, and SW libraries, respectively. The community diversity and richness is lowest in the bitumen (Table 1). Community richness is similar in both water bodies, although the $H_{shannon}$ index evidences a higher diversity in the surface water. The higher diversity is associated with drastically different bacterial communities. Sequences of the SW library are composed of equal fractions of alpha-, beta-, epsilon and gammaproteobacteria (Fig. 2), while in the UW and B libraries are dominated by *Epsilonproteobacteria* (80.4 and 83.9% respectively). Most *Epsilonproteobacteria* sequences at Puy-de-la-Poix are associated with the genus *Sulfurimonas*, which represent 56.9% and 63.3% of the total bacterial diversity in the B and UW libraries, respectively. The remaining diversity of *Epsilonproteobacteria* is represented by *Sulfurovum* and *Arcobacter* (12.1% and 10.3% of the total bacterial diversity respectively in the B library, and 8.1% of the total bacterial diversity for both groups in the UW libraries). A single *Sulfurimonas* OTU, e.g. OTU 11 (Table 2, Fig. 2) dominates *Epsilonproteobacteria* diversity and accounts for 30 and 35% of *Epsilonproteobacteria* in the UW and B libraries (Fig. 2), but is not found in the SW library. OTU 11 is most closely related to the type species of the *Sulfurimonas gotlandica* species [14]. Four OTUs were associated with the *Sulfurovum aggregans* type strain Monchim33, which has been isolated from a hydrothermal vent system [15]. The *Sulfurovum* OTUs are related to environmental clones of very diverse origins, a soil, a sediment mesocosm, a bivalve symbiont or an acid cave. *Arcobacter* sequences (6 OTUs) represent a minor, but

Table 1
Community richness (S_{chao1}) and community diversity ($H_{Shannon}$).

	S_{Chao1}	$H_{Shannon}$
B	60	2,57
UW	306	2,78
SW	335	3,35

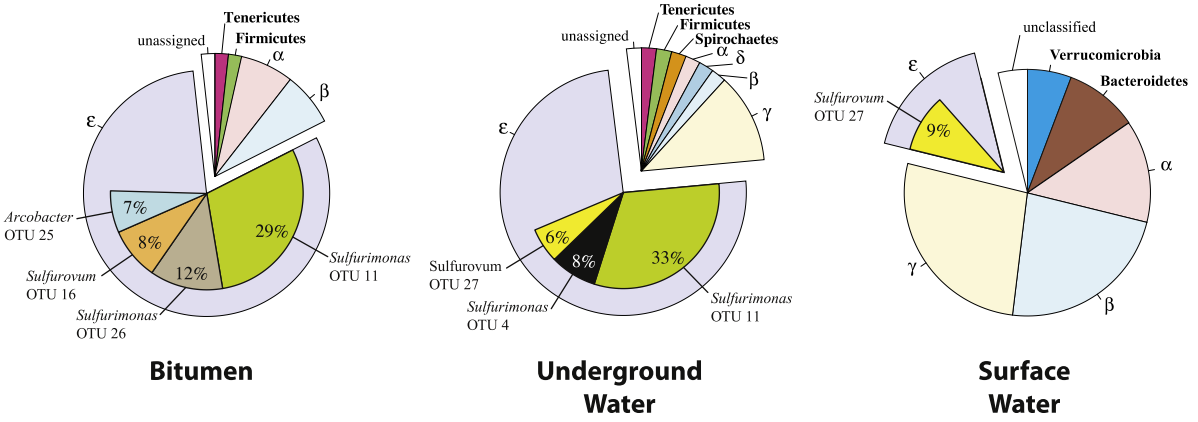

Fig. 2. Total bacterial diversity at the Puy-de-la-Poix. Major *Epsilonproteobacteria* OTUs and their taxonomy, are shown as inserts within the *Epsilonproteobacteria* (purple).

Table 2
Diversity and taxonomic position of the epsilonproteobacterial sequences.

OTU	Reference clone	B	UW	SW	Identification	Closest cultivated isolate[a]		Closest environmental sequence[b]			
						Species and strain name	Sampling site	% ID	Accession	% ID	Sampling site
1	UW-31		1		*Arcobacter*	*Arcobacter cloacae* SW28-13	Sewage station	98.9			
8	B-31	1			*Arcobacter*	*Arcobacter marinus* CL-S1	Seawater	99.4	AY569293	99.9	Hot spring
9	UW-55		1		*Arcobacter*	*Arcobacter bivalviorum* F4	Mussels	92.7	FR666865	97.3	Cold seep
12	UW-35		1		*Arcobacter*	*Arcobacter venerupis* F67-11	Mussels	95.8	HQ538628	98.1	Sludge
14	B-2	1			*Arcobacter*	*Arcobacter nitrofigilis* DSM 7299	Marshplant roots	98.6	AY704399	99.6	Crustal fluids
25	B-9	4			*Arcobacter*	*Arcobacter venerupis* F67-11	Mussels	97.1	EU617863	98.3	Yellow Sea sediment
2	UW-36		1		*Arcobacter*	*Arcobacter ellisii* F79-6	Mussels	92.7	EU265974	97.2	Lake sediment
10	UW-58		1		*Sulfuricurvum*	*Sulfuricurvum kujiense* DSM 16994	Oil polluted water	98.7			
6	UW-89		1		*Sulfurimonas*	*Sulfurimonas sp.* MA01	Marine sediment	97.0	AB240698	97.8	Sediments
4	UW-45	1	4		*Sulfurimonas*	*Sulfurimonas autotrophica* DSM 16294	Marine sediment	95.1	AY922199	98.6	Various
7	UW-33		1		*Sulfurimonas*	*Sulfurimonas autotrophica* DSM 16294	Marine sediment	93.6	EF219001	95.9	Hydrothermal vent
13	B-18	1			*Sulfurimonas*	*Sulfurimonas denitrificans* DSM 1251	Hydrothermal vent	91.2	EU570862	93.3	Lake sediment
24	B-32	3	1		*Sulfurimonas*	*Sulfurimonas denitrificans* DSM 1251	Hydrothermal vent	95.1	FJ717065	97.5	Mesocosm
29	B-1	1			*Sulfurimonas*	*Sulfurimonas denitrificans* DSM 1251	Hydrothermal vent	96.1	UOU46506	98.2	Oil field
22	B-54	1		1	*Sulfurimonas*	*Sulfurimonas denitrificans* DSM 1251	Hydrothermal vent	94.0	DQ112511	95.8	Mudflat sediment
3	UW-88		1		*Sulfurimonas*	*Sulfurimonas gotlandica* GD1	Pelagic redoxcline	95.1	AB478650	97.7	Microbial mat
5	UW-41		1		*Sulfurimonas*	*Sulfurimonas gotlandica* GD1	Pelagic redoxcline	85.6	FJ628185	89.1	Lake sediment
11	B-22	17	16		*Sulfurimonas*	*Sulfurimonas gotlandica* GD1	Pelagic redoxcline	94.8	AB478672	96.6	Microbial mat
18	UW-84		1		*Sulfurimonas*	*Sulfurimonas gotlandica* GD1	Pelagic redoxcline	93.5	FJ037617	97.0	Iron-ixodizing biofilm
19	B-7	2	1		*Sulfurimonas*	*Sulfurimonas gotlandica* GD1	Pelagic redoxcline	94.6	FJ437845	98.0	Lake sediment
20	SW-37			1	*Sulfurimonas*	*Sulfurimonas gotlandica* GD1	Pelagic redoxcline	94.8	FJ437845	97.7	Lake sediment
21	UW-52		1		*Sulfurimonas*	*Sulfurimonas gotlandica* GD1	Pelagic redoxcline	94.5	AB478672	97.7	Microbial mat
26	B-16	7			*Sulfurimonas*	*Sulfurimonas paralvinellae* GO25	Polychaete nest	95.1	AB247901	96.4	Hydrothermal vent
28	UW-29		1		*Sulfurimonas*	*Sulfurimonas gotlandica* GD1	Pelagic redoxcline	91.3	GQ261791	94.0	Deep sea
23	SW-32			1	*Sulfurospirillum*	*Sulfurospirillum arsenophilum* MIT-13		98.6			
16	B-13	5			*Sulfurovum*	*Sulfurovum aggregans* Monchim33	Hydrothermal vent	95.9	GU583971	97.6	Mangrove Soil
17	B-12	1	1	1	*Sulfurovum*	*Sulfurovum aggregans* Monchim33	Hydrothermal vent	96.8	FJ717160	98.6	Mesocosm
27	UW-37		3	5	*Sulfurovum*	*Sulfurovum aggregans* Monchim33	Hydrothermal vent	95.3	EF467592	100	Acid cave
15	B-26	1			*Sulfurovum*	*Sulfurovum aggregans* Monchim33	Marine sediment	94.1	EU487914	95.9	Bivalve-symbiont
	L12				*Sulfurospirillum*	*Sulfurospirillum multivorans* DSM 12446	Activated sludge	93.5			

OTU: OTU number. Reference clone, name of the reference sequence representing the OTU; B, UW, SW, number of sequences in each OTU for bitumen, underground water and surface water sample libraries repectively.
[a] Taxonomy, strain name and percent homology of the *16S rRNA* gene sequence from the closest cultivated organisms.
[b] Accesssion number and percent identity of the closest *16S rRNA* gene sequence from uncultured organisms. This information is only provided when sequence more closely related than the cultivated isolate.

significant, component of the bacterial diversity (10% and 8% for the B and UW libraries respectively). The last two OTUs belong to the *Sulfuricurvum* and *Sulfurospirillum* genera (Table 2). There is a large overlap between the bacterial populations of the UW and B libraries, which is consistent with a common origin of the two populations, and the probability that *Epsilonproteobacteria* were present in the zone of contact of the oil and water. Indeed, such large prevalence of *Epsilonproteobacteria* has been reported previously at the oil/water interface in other geological settings such as a highly degraded oil reservoir or an oil spill from a storage tank [5,6]. The absence of a bacterial diversity related to known bacterial fermenters or syntrophs at the Puy-de-la-Poix, and the low level of sulfate-reducers suggested that the degradation of the organic matter of the oil and sulfate reduction may not be occurring at a significant level at the Puy-de-la-Poix, and that microbial degradation occurred during the transit of the oil to the surface.

Isolation of cultivable *Epsilonproteobacteria*. Obtaining cultivable *Epsilonproteobacteria* from the Puy-de-la-Poix was a prerequisite to assay their metabolic abilities.

Six pure cultures were obtained using the *Thiomicrospira* mineral base medium supplemented with either acetate or lactate, and nitrate as an electron acceptor. The *16S rDNA* gene sequences of the six isolates were almost identical (> 99% sequence identity), defining a single OTU closely related to *Sulfurospirillum multivorans* (Clone L12, Table 2 and Fig. 3). The strains isolated here are closely related, but do not belong to OTU 23 (SW library, Table 2, Fig. 3). Microscopic observations of the isolates revealed curved or helical rods. The cells were non-motile. Growth was obtained with acetate and lactate, but not pyruvate, formate or sugars in the presence of nitrate under anaerobic conditions. No growth was observed on sulfate or thiosulfate as electron acceptors. As expected, no growth occurred under aerobic conditions. Typical growth was low, reaching ca. 10^7 cells/ml within two weeks. The apparent doubling time of strain L12 is ca. 24 h. This doubling time is extremely long for the *Sulfurospirillum* species, for which the doubling time is usually in the order of a few hours. Growth yields were also extremely low. Both observations indicate that the growth medium used to isolate strain L12, although supporting growth, is lacking some important components, which we have been unable to identify thus far. No pure culture belonging to the major *Epsilonproteobacteria* clades (Fig. 3) could be isolated.

Role of *Epsilonproteobacteria* at Puy-de-la-Poix. Only two OTUs observed at the Puy-de-la-Poix are related to isolates from oil/water interfaces: OTU 10 is related to *Sulfuricurvum kujiense*, a species isolated from an oil storage tank [16]. The second OTU is related to the CVO strain, which has been isolated from the Coleville oil field [17]. Both *S. kujiense* and CVO are able to reduce nitrate with the concomitant oxidation of sulfur compounds. Both organisms have been found to dominate the bacterial diversity at their isolation sites, although they were unable to degrade the organic matter of the oil. Therefore, their role in these particular ecosystems, and the source of carbon supporting their growth remained unclear. Most of the *Epsilonproteobacteria* at Puy-de-la-Poix were most closely related to *Sulfurimonas gotlandica* [14]. *S. gotlandica* is a sulfur oxidizer, nitrate-reducing chemolithoautotrophic bacterium that has been isolated from the deep-sea abyssal sediments of the Gotland depth. Similarly to *S. kujiense* and strain CVO, *S. gotlandica* is unable to use small organics, such as pyruvate, lactate of formate, but uses dissolved carbonates to support its growth, a phenomenon known as high dark CO_2 fixation [18]. Thus, it is very unlikely that *Epsilonproteobacteria* at Puy-de-la-Poix may be involved in the breakdown of the complex organic matter of the tar. High dark CO_2 fixation is important in the suboxic to sulfidic transition zones of aquatic pelagic redox zones, as in the Black Sea and the Baltic Sea from which *S. gotlandica* has been isolated [14,18–21]. There are enough carbonates in the water at Puy-de-la-Poix to support high dark CO_2 fixation by chemolithoautotrophic *Epsilonproteobacteria* (Table S2) oxidizing sulfur and reducing nitrates. Thus, the dominance of *Epsilonproteobacteria* could be explained by the refractory nature of the residual organic matter composing the tar, which would limit the access of fermenters and

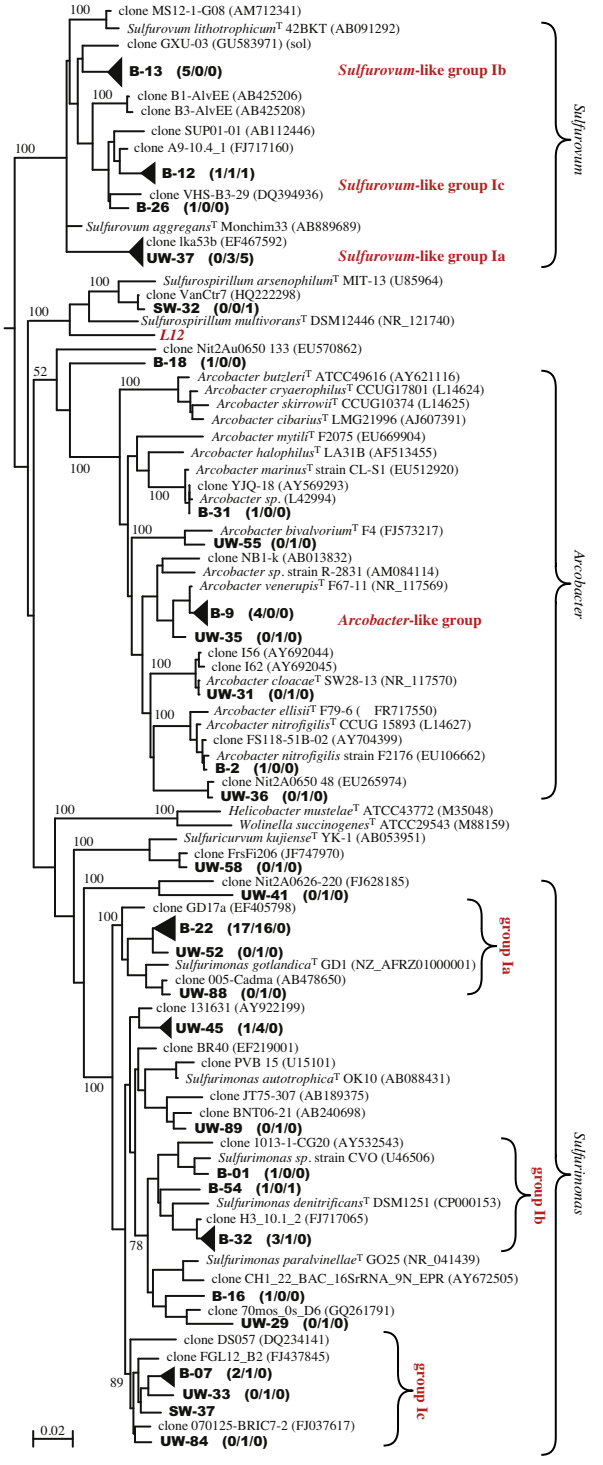

Fig. 3. Phylogenetic tree of the *16S rDNA* gene sequences from environmental clones of the UW, SW and B libraries and reference sequences from the databases. Clones from the present study are shown in boldface type. The topology was obtained by comparing nearly full-length sequences of the *16S rRNA* gene using the maximum likelihood approach with PhyML. Similar tree topologies were produced using other tree reconstruction algorithms. The scale bar represents the number of changes per nucleotide position. Filled triangles were substituted to individual clones for some clusters of sequences to improve readability of the tree. The height of the triangles is drawn to scale with the diversity of each group.

syntrophs to carbon and energy sources, while *Epsilonproteobacteria* could rely on reduced sulfur compounds and CO_2 fixation for growth. *Epsilonproteobacteria* could reduce sulfur compounds of the oil, but more probably dissolved reduced sulfur species, such as hydrogen sulfide, which are present in the water due to the hydrothermal activity of this geologic province.

4. Conclusions

The bacterial diversity at Puy-de-la-Poix shows an unexpected domination of *Epsilonproteobacteria*. The lack of known sulfate-reducers or syntrophs is a clear indication that oil mineralization to methane does not occur at this site. The abundance of *Epsilonproteobacteria* indicates that reduced sulfur species are the major energy source, ruling out significant degradation of the oil organic matter under anaerobic conditions. Our observations do not rule out the possibility of aerobic microbial oil degradation, in contact with surface waters, or when oil overflows in the nearby fields. The presence of large populations of *Epsilonproteobacteria* at the water/oil interface of different geological settings (oil reservoirs, oil contaminated waters or natural seep) is a clear indication that this interface constitutes a favorable ecological niche for these organisms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.crvi.2016.10.001.

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