Preservation of ancestral Cretaceous microflora recovered from a hypersaline oil reservoir

Grégoire Gales1,2, Nicolas Tsesmetzis3, Isabel Neria2, Didier Alazard2, Stéphanie Coulon4, Bart P. Lomans5, Dominique Morin4, Bernard Ollivier2, Jean Borgomano1 & Catherine Joulian4

Microbiology of a hypersaline oil reservoir located in Central Africa was investigated with molecular and culture methods applied to preserved core samples. Here we show that the community structure was partially acquired during sedimentation, as many prokaryotic 16S rRNA gene sequences retrieved from the extracted DNA are phylogenetically related to actual Archaea inhabiting surface evaporitic environments, similar to the Cretaceous sediment paleoenvironment. Results are discussed in term of microorganisms and/or DNA preservation in such hypersaline and Mg-rich solutions. High salt concentrations together with anaerobic conditions could have preserved microbial/molecular diversity originating from the ancient sediment basin wherein organic matter was deposited.

Subsurface environments harbor 1/10 to 1/3 of global living biomass, thus playing an important role in biogeochemical cycling of elements1–2. Subsurface oil-bearing reservoir rocks have been extensively studied during the last century with respect to the critical economic value of hydrocarbons. The probable rarefaction of this fossil resource and the possibility to sequestrate CO2 within depleted oil fields3 stimulate multidisciplinary scientific investigations including microbiology. Whereas the presence of microorganisms in oil reservoirs was recognized earlier4, the reality of their effective in situ activity remains elusive1. Biodegradation of organic matter in sedimentary rocks contributes to the biogeochemical cycling of carbon1 and strongly impacts the quality and exploitation of hydrocarbons1. Oil reservoirs are nutrient-depleted environments, especially lacking of phosphorus and nitrogen. They contain an excess of reduced electron donors (hydrocarbons) but a shortage of electron acceptors (e.g., nitrate, oxygen…) and are considered as anaerobic environments5–7. We characterized the indigenous microbiota of a hypersaline oil reservoir by molecular and cultural analyzes of rock samples, rather than from fluid samples (e.g., production fluid from well heads of oil producing wells) possibly affected by greater exogenous microbial contamination8. The core was collected from an onshore oil reservoir in Central Africa, before oil exploitation and any fluid injection.

Results

Core chemistry and mineralogy. The core was sampled at 1153–1154 m depth in a lower Cretaceous sandstone, underlying the transgressive Gamba Formation and thick salt deposits9. Reservoir pressure and temperature were respectively 12 Mpa and 43 °C. These sediments are associated to the South Atlantic Aptian salt deposition contemporary of the basin creation resulting from the break-up between Africa and South America10. The unconsolidated sandstone consisted in quartz-felspar grains coated with green clays and salt crystals, without stratification. NaCl crystals were very abundant on the grain surfaces and within the clay coatings (Fig. 1). Intergranular cements, such as quartz, were absent. Most of the grains were coated by clays (smectite, chlorite, illite), which probably inhibited the development of quartz overgrowth in the intergranular pore space. Local chemical analyzes and X-ray diffraction on the bulk sample and on separated clays confirmed the nature of the minerals. Moreover, as chlorite minerals from the inner core were not oxidized, anaerobic conditions during coring, sample preservation (by aluminium barrel, freezing in liquid nitrogen), shipping to and transferring at
the laboratory should have been preserved. In this respect, our sampling conditions should not have affected the composition of the existing microbial diversity of the inner section (6 cm) of the 10 cm core. Chemical analyzes are summarized in Table S1. Formation waters had salinity close to saturation and the core itself contained above 13 mg/g of Na\(^+\) (supplementary material, Table S1).

Bacterial and archaeal molecular diversities. 16S rRNA gene surveys, including classical clone libraries analyses (case of Bacteria and Archaea) and high throughput sequencing methods (case of Bacteria) were conducted on DNA extracted directly from the core (Tables 1 and 2). The rarefaction curves from cloning (supplementary material Fig. S2) showed that the Rabi core harbored low archaeal diversity as saturation was obtained. But the Rabi core was inhabited by a more diverse bacterial community than the one estimated by the clone library as the rarefaction curve was not saturated, highlighting the need to perform deeper 16S rRNA gene sequencing to assess it.

Clone libraries analyses. Most of the bacterial OTUs (Operational Taxon Units) were related to aerobes and facultative anaerobes known to degrade oil compounds. From 92 clones analyzed, the library was dominated (50%) by OTU related to aerobic *Ochrobactrum* spp., of which some strains were found in crude oil or reported to degrade aliphatic and aromatic hydrocarbons\(^1\). Three OTUs (13% of the library) were related to facultative anaerobic, halophilic marine species of the *Halomonas* genus with *H. shengliensis* being reported to degrade crude oil\(^2\). A fourth *Halomonadaceae* OTU was related to the halophilic strain *Chromohalobacter salexigens*, formerly *Halomonas elongata*. As well, minor OTUs were related to aerobes of the genera *Burkholderia* (4.3% of the library) and *Microbacterium* (1.1% of the library) of which species are capable to aerobically degrade crude oil\(^3,4\).

Besides the *Halomonas* spp., other OTUs were also related to halophilic microbes, a physiological trait compatible with the *in situ* salinity. Indeed, the second most dominant OTU (19.6%) of the library was related to the marine genus *Halolactibacillus* recognized as facultative anaerobic halophiles\(^5\). We retrieved a member of the *Firmicutes* related to the *Halanaerobium* genus (4.3% of the library); members of this genus have been frequently recovered from oil reservoirs\(^6,7\) and some of them inhabit sebkhas similar to the paleoenvironment of Rabi sandstone\(^8\). We also report the occurrence of members of the genus *Orenia*, family *Halobacteroidaceae*. Interestingly, few representatives of this family have been retrieved by molecular approaches from oil reservoirs so far\(^9,10\). They include phylogenetic relatives of the genus *Orenia* recovered from hot reservoirs\(^10\) where their *in situ* activity is highly questionable. We found only one clone phylogenetically related to the anaerobic, thermophilic and halophilic genus *Geotoga*, order *Thermotogales*. Presence of members of this order is also recurrent in oil fields\(^10,11\).

Within the 36 archaeal clones analyzed, the large majority of OTUs belonged to the *Halobacteriaeae* family regrouping mainly extreme aerobic halophiles. Similar microorganisms have been already isolated from high saline reservoirs\(^12,13\). The library is dominated (>30%) by OTUs related to *Halococcus hamelinensis* and *Haloplanus natans* isolated from saline environments, respectively stromatolites from Shark Bay, Australia\(^14\) and the Dead Sea\(^15\). Then, 16.7% of the OTUs belonged to the *Halorhabdus* genus; among the two described species within this

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Figure 1. SEM image of the sand. Quartz-felspar grains are coated with clays, salt (NaCl) crystalized on the grain surfaces.
### Members of the Bacteria domain - Proteobacteria

| Clone   | % in library | Closest related microorganism (% of similarity by blast) | Lineage                                      | Main metabolic traits of the related microorganisms                      |
|---------|--------------|----------------------------------------------------------|---------------------------------------------|--------------------------------------------------------------------------|
| MIG-B2  | 50.0         | Ochrobactrum sp. (99)                                      | Rhizobiales; Brucellaceae                  | Aerobic, mesophilic, possible degradation of crude oil by some Ochrobactrum spp. |
| MIG-B13 | 7.6          | Halomonas venusta (99)                                     | Oceanospirillales; Halomonadaceae          | Facultative anaerobic, halophilic, marine bacterium; possible degradation of crude oil by some Halomonas spp. |
| MIG-B23 | 4.3          | Halomonas meridiania (99) Halomonas aquamarina (99)       | Oceanospirillales; Halomonadaceae          | Facultative anaerobic, halophilic, marine bacterium; possible degradation of crude oil by some Halomonas spp. |
| MIG-B19 | 4.3          | Burkholderia ferrariae (99) Burkholderia silvanlantica (99) | Burkholderiales; Burkholderiacae           | Aerobic, mesophilic; possible degradation of alkanes and aromatic hydrocarbons by some Burkholderia spp. |
| MIG-B18 | 2.2          | Rhizobium loti (99)                                        | Rhizobiales; Phyllobacteriaceae            | Aerobic and mesophilic bacterium                                         |
| MIG-B1  | 1.1          | Halomonas phoceae (97) Halomonas xinjiangensis (97)       | Oceanospirillales; Halomonadaceae          | Facultative anaerobic, halophilic, marine bacterium; possible degradation of crude oil by some Halomonas spp. |
| MIG-B9  | 1.1          | Chromohalobacter salxigenus (99)                          | Oceanospirillales; Halomonadaceae          | Aerobic, moderate halophilic, marine bacterium                            |

### Members of the Bacteria domain - Firmicutes, Actinobacteria, and Thermotogae

| Clone   | % in library | Closest related microorganism (% of similarity by blast) | Lineage                                      | Main metabolic traits of the related microorganisms                      |
|---------|--------------|----------------------------------------------------------|---------------------------------------------|--------------------------------------------------------------------------|
| MIG-B16 | 19.6         | Halolactibacillus halophilus (98) Halolactibacillus miuriensis (98) | Firmicutes; Bacillaceae                     | Facultative anaerobic, halophilic and alkaliphilic marine lactic acid bacterium |
| MIG-B8  | 4.3          | Halanaerobium fermentans (99)                            | Firmicute; Halanaerobiales; Halanaerobiaceae | Anaerobic, halophilic fermentative bacterium                             |
| MIG-B25 | 2.2          | Orenia marismortui (95)                                   | Firmicute; Halanaerobiales; Halobacteroidaceae | Anaerobic, halophilic fermentative bacterium                             |
| MIG-B42 | 1.1          | Orenia marismortui (95)                                   | Firmicute; Halanaerobiales; Halobacteroidaceae | Anaerobic, halophilic fermentative bacterium                             |
| MIG-B15 | 1.1          | Microbacterium paraoxydans (99)                          | Actinobacteria Actinomycetales; Microbacteria | Aerobic, mesophilic; possible degradation of crude oil by some Microbacterium spp. |
| MIG-B3  | 1.1          | Geotoga aestuarianus (99)                                 | Thermotogales; Thermotogaceae              | Anaerobic, thermophilic fermentative bacterium                            |

### Members of the Archaea domain - Euryarchaeota

| Clone   | % in library | Closest related microorganism (% of similarity by blast) | Lineage                                      | Main metabolic traits of the related microorganisms                      |
|---------|--------------|----------------------------------------------------------|---------------------------------------------|--------------------------------------------------------------------------|
| MIG-ARCH1 | 33.3        | Halococcus halodurans (99)                                | Halobacteriales                             | Aerobic, extreme halophile                                               |
| MIG-ARCH6 | 30.6        | Haloplanus natans (98)                                    | Halobacteriales                             | Aerobic, extreme halophile                                               |
| MIG-ARCH2 | 16.7        | Halorhodobacterium halophilus (97) Halorhodobacterium utahensis (97) | Halobacteriales                             | Facultative anaerobic (microaerobic) extreme halophile                  |
| MIG-ARCH27 | 16.7      | Halarchaeum acidiphilum (97)                              | Halobacteriales                             | Aerobic, extreme halophile                                               |
| MIG-ARCH10 | 2.8         | Thermococcus (77)                                         | Thermococcales                              | Anaerobic hyperthermophile                                               |

### Table 1. Diversity of Archaea and Bacteria retrieved in the core by 16S rRNA gene surveys. Retrieved environmental 16S rRNA gene sequences are available under Genbank accession numbers JQ690672 to JQ690688.

The most abundant OTU in the core (Table 2) was related to the marine, halophilic, facultative anaerobe *Halomonas* (27.9%). Some *Halomonas* spp. are reported to be involved in the degradation of crude oil and others are reported to originate from highly saline environments. Besides *Halomonas*, a high proportion of OTUs was related to the thermophilic sulfate-reducing bacterium *Desulfonauticus*, family *Desulfohalobiaciae* (11%). *Desulfonauticus* spp. were previously isolated from oil production water and deep sea hydrothermal vents. We have also identified a number of OTUs related to the genus *Pseudomonas* (10.1%) comprising species possibly degrading aromatic hydrocarbons both aerobically and anaerobically. Another genus that matched many of the produced OTUs was the facultative anaerobic, halophilic marine bacterium *Marinobacter* (7.1%) members of which are capable to also degrade petroleum hydrocarbons.

In addition to *Desulfonauticus* mentioned above, we identified in moderate amounts some OTUs that are related to anaerobic mesophilic sulfate and/or sulfur reducers such as *Desulfovibrio* (0.8%), order *Desulfovibrionales* (2.9%), *Pelobacter* (0.7%), as well as to anaerobic thermophiles (e.g. *Thermovirga*, 0.4%). Members of these taxa...
| % in library | Closest relative* | Lineage | Main metabolic traits of the related microorganisms |
|-------------|------------------|---------|---------------------------------------------------|
| 27.9        | Halomonas        | Gammaproteobacteria; Oceanospirillales; Halomonadaeae | Facultative anaerobic, halophilic, marine bacterium; possible degradation of crude oil by some Halomonas spp. |
| 11.0        | Desulfonaicus    | Deltaproteobacteria; Desulfovibrionales; Desulphohalobias | Anaerobic, thermophilic sulfate-reducing bacteria isolated from oil production water and deep-sea hydrothermal vents |
| 10.1        | Pseudomonas      | Gammaproteobacteria; Pseudomonadales; Pseudomonadaeae | Aerobic, known to degrade aromatic hydrocarbons |
| 7.1         | Marinobacter     | Gammaproteobacteria; Alteromonadales; Alteromonadaeae | Facultative anaerobic, halophilic, marine bacterium; possible degradation of crude oil by some Marinobacter spp. |
| 6.1         | n.d.             | Gammaproteobacteria; Enterobacteriales; Enterobacteriaeae | Facultative anaerobic, many of them are nitrate reducers under anaerobic conditions |
| 5.7         | n.d.             | Bacteroidia; Bacteroidales; | |
| 4.2         | n.d.             | Clostridia; Clostridiales; Clostridiacea | Primarily anaerobic; many of its genera are fermenters |
| 2.9         | n.d.             | Deltaproteobacteria; Desulfovibrionales; | Obligatory anaerobic, mesophilic or moderately thermophilic sulfate reducers |
| 2.1         | n.d.             | [Cloacamonaceae]; [Cloacamonales]; MSBL8 | Affiliated with Spirochaetes, anaerobic, halotolerant |
| 1.4         | Oceanobacillus   | Bacilli; Bacillales; Bacillaceae | Halotolerant, alkalophilic, mesophilic, facultative anaerobic or strictly aerobic bacterium |
| 1.2         | n.d.             | Gammaproteobacteria; Oceanospirillales; Oceanospirillaceae | Most genera are halophilic, aerobic or facultative anaerobic, chemoorganotrophs |
| 1.1         | n.d.             | Betaproteobacteria; Burkholderiales; Comamonadaeae | Aerobic organotrophs, anaerobic denitrifiers and Fe(III)-reducing bacteria, hydrogen oxidizers, photoautotrophic and photoheterotrophic bacteria, and fermentative bacteria, members are known to degrade hydrocarbons |
| 0.9         | Ralstonia        | Betaproteobacteria; Burkholderiales; Oxalobacterales | Aerobic, can reduce nitrate anaerobically, found in both soil and water, used in bioremediation, chlorinated aromatic compounds degrader |
| 0.8         | Desulfovibrio    | Deltaproteobacteria; Desulfovibrionales; Desulfobacteriaceae | Anaerobic, sulfate-reducer |
| 0.7         | Pelobacter       | Deltaproteobacteria; Desulfobacteriales; Pelobacteriales | Strictly anaerobic, Fe(III) or S(0) reducer |
| 0.6         | Halanaerobium    | Clostridia; Halanaerobiales; Halanaerobiumaceae | Anaerobic, halophilic fermentative bacterium |
| 0.4         | n.d.             | Flavobacteria; Flavobacteriales; Flavobacteriaceae | Aerobic, known to degrade aromatic hydrocarbons |
| 0.4         | Thermovigia      | Synergistia; Synergistales; Thermovigiaeae | Aerobic, known to degrade hydrocarbons |
| 0.4         | Marinilactibacillus | Bacilli; Lactobacillales; Lactobacteriaceae | Aerobic, known to degrade hydrocarbons |
| 6.2         | Unassigned       | | |
| 9.0         | Other            | | |

Table 2. Diversity of Bacteria retrieved from the core using Illumina sequencing of the 16S rRNA gene. Sequences are available at BioProject ID PRJNA301727; n.d.: not determined.

have been already successfully isolated from petroleum hydrocarbon associated environments30–32. Additionally, fermentative bacteria such as those belonging to the genus Halanaerobium (0.6%), as already mentioned in the bacterial clone library, and the Clostridiae family (4.2%) were also found to have representative OTUs in the dataset. Halanaerobium has been previously found in formation water samples from an oil reservoir30 whereas members of the Clostridiae family are thought to be using hydrocarbon intermediates to generate organic acid precursors under sulfate, methanogenic or iron reducing conditions34–36. Other genera related to the producers OTUs include Oceanobacillus (1.4%), Ralstonia (0.9%), Marinilactibacillus (0.3%) as well as members of the families Enterobacteriaceae (6.1%), Clone MSBL8 (2.1%), Oceanospirillaceae (1.2%), Comamonadaceae (1.1%), Flavobacteriaceae (0.4%). Finally, OTUs related to members of the order Bacteroidales were also found in the sample at a relative high number (5.7%). The significance of many of the above taxa to the Rabi’s in situ reservoir conditions is still unknown as many of them are either still poorly characterized in the literature.

**Culture experimentation.** Neither sulfate reducers, nitrate reducers, sulfide oxidizers nor hydrogenotrophic and methylotrophic methanogens were encountered. Only fermentative Bacteria, and specially members of the phylum Firmicutes, representatives of the Bacillaceae and Halobacteriaceae families (Table 3) were cultivated. Strains belonging to the genus Halanaerocella petrolearia of the family Halobacteriaceae were isolated; this species is perfectly adapted to this environment as it grew anaerobically at high saline concentrations37.
the successful isolation of Archaea in environments like soils, followed by a PCR artefact. Furthermore, no Archaea may be due to an external contamination of the core, the genus detected in the clone library and not by the culture-dependent method or the Illumina sequencing, its presence in the Rabi core DNA of sufficient yield and quality for Illumina sequencing. Even if acknowledging the introduction of fragments that have been degraded. A 2-step approach was however necessary to produce amplicons from the Rabi core DNA, as sequences retrieved from Rabi core. However, contrary to these environments, the Rabi core is anaerobic and not suitable to sustain viability of these hyperhalophilic aerobic archaeons. It could also be due to the medium formulations or to specific niches being sampled. Discrepancies between culture-dependent and -independent methods targeting biological diversity are common in microbiological studies. It could reflect our inability to mimic natural environmental conditions sufficiently to sustain growth, and/or our inability to extract and amplify correctly DNA from natural and industrial environments. The retrieved strict anaerobes as well as the facultative ones show traits compatible with the Rabi environment (ability to develop in the absence of oxygen and at high salinity) and have for some of them already been retrieved from oil reservoirs (e.g. Halobacteriaceae, Oreniaceae and Haloanaerobiaceae, as sequences retrieved from old and salty environments). Isolates belonged to the same family and genus, as sequences retrieved from Rabi core. However, contrary to these environments, the Rabi core is strictly anaerobic and not suitable to sustain viability of these hyperhalophilic aerobic archaeons. It could also be due to the medium formulations or to specific niches being sampled. Discrepancies between culture-dependent and -independent methods targeting biological diversity are common in microbiological studies.

### Discussion

Clone library analysis and Illumina sequencing results gave two different pictures of molecular bacterial diversity. Expected strict anaerobes are found essentially from the Illumina sequencing where they account for a larger part of the diversity (27.6%) than in the clone library (8.7%). But the originality of both results is the abundance of aerobic and facultative anaerobic microorganisms in a strictly anaerobic environment. As expected when considering the number of reads, Illumina sequencing showed a greater diversity than the clone library. The applied protocol included a nested-PCR which, in addition to increase specificity, will also increase sensitivity. Sequences initially poorly amplified may be enriched after the nested-PCR step, resulting in a larger diversity retrieved by sequencing. As DNA used for high throughput sequencing came from the same core but after a longer storage time, we may also infer that Illumina sequencing platform, which amplify shorter fragments (440 bp) than one used for the construction of the clone library (1400 bp), has found target sequences on shorter genomic DNA fragments that have been degraded. A 2-step approach was however necessary to produce amplicons from the Rabi core DNA of sufficient yield and quality for Illumina sequencing. Even if acknowledging the introduction of biases, according to in-silico primer analysis, we estimated a limited reduction of coverage of the Bacteria lineages, especially if a one mismatch is allowed between primer and template (data not shown).

### Table 3. Microbial diversity of isolates originating from the hypersaline oil reservoir and their condition of isolation.

| Isolates | Total | Closest phylogenetic relative | % blast similarity | Condition of isolation |
|----------|-------|------------------------------|-------------------|-----------------------|
| RAH4 and FERM001 | 2 | *Thalassobacillus devorans* | 99–100 | 250 g.L⁻¹ NaCl pH 8.8 anaerobiosis (Table S2) |
| FERM003 and AER001 | 2 | *Bacillus circulans* | 99–100 | 120 g.L⁻¹ NaCl pH 8.8 anaerobiosis (Table S2) |
| 1D4 | 1 | *Orenia marismortui* | 99 | 250 g.L⁻¹ NaCl pH 8.8 anaerobiosis (Table S2) |
| Deha1, Deha2 and Deha3 | 3 | *Halanaerocella petrolearia* (Strain S200) | 100 | 250 g.L⁻¹ NaCl pH 8.8 anaerobiosis (Tables S3 and S6) |
| Alpha | 1 | *Halobacillus trueperi/dabanensis* | 99 | 250 g.L⁻¹ NaCl pH 7.5 anaerobiosis (Table S2) |

However, it was not recovered from the molecular surveys. Other isolates were also related to highly saline shallow or surface environments. It is the case of strains closely related to (i) *Halobacillus trueperi* commonly found in Tunisian sebkhas or chotts, (ii) *Orenia marismortui*, which is described as adapted also to highly saline surface environments and degrading complex organic compounds and (iii) *Thalassobacillus devorans*, formerly isolated from hypersaline habitats and able to oxidize aromatic compounds. Within archaeal clone library, mainly hyperhalophilic aerobic *Archaea* and facultative anaerobes appeared to be dominant from molecular studies, although the core was essentially anaerobic. However, despite repeated cultivation efforts, none of these archaeons were isolated thus suggesting that they are not metabolically active in the oil field, most probably because of strict anaerobic conditions prevailing in situ and limited access to organic matter restricted to hydrocarbons, together with the lack of suitable electron acceptors possibly used by some of these microorganisms (e.g. nitrate, fumarate).

### Table 3. Microbial diversity of isolates originating from the hypersaline oil reservoir and their condition of isolation. 16S rRNA gene sequences of isolates are available under Genbank accession numbers JQ690689 to JQ690697.
nitrate or fumarate as terminal electron acceptors. In this respect, unknown types of anaerobic metabolism to be performed by these microorganisms cannot be excluded.

Are they of ecological significance in this ecosystem or do they reflect the existing biodiversity during the sediment settling in the basin before the process of oil filling resulting in a long-term dormancy? Two hypotheses could afford for the detection of aerobic microorganisms in an anaerobic, highly saline environment. First, microorganisms are not active and DNA has been preserved over millions of years; second, microbial activity is scarce and community has been preserved in a slow-growing state for millions years.

DNA preservation over geological times has been a matter of debate in the subsurface microbiology. Although bacterial DNA recovery from historical samples can lead to full genome sequencing of ancient bacteria, there are nowadays few studies that report the effectiveness of very ancient (at least prior to the Holocene) microbial DNA recovery. If fully hydrated DNA spontaneously decays over only hundreds of years, extension of the half-life of intact DNA can be achieved by low temperature, high ionic strength, anoxic conditions and protection from enzymatic degradation. High salt concentrations could thus be a great factor of biomolecule preservation. The salt-saturated solution filling the reservoir pores may explain the microbial diversity and the possible DNA preservation within the oil reservoir. Presence of physiological concentrations of K+ and Mg2+ strongly reduce thermal degradation of DNA. Protective effect of salt on biological macromolecules such as tRNA is demonstrated at NaCl concentration between 0.5 and 3 M. Effects of the extremely chaotropic and soluble MgCl2 salt have been studied in the deep sea hypersaline lake Discovery (eastern Mediterranean). MgCl2 concentration above 1.26 M inhibited the growth of all microorganisms taken from this environment. MgCl2 at high concentrations not only denatures macromolecules, but also preserves the more stable ones, like DNA. Other results confirmed the preservation and the transforming power of DNA when solubilized in this type of brine, thus being prone to PCR amplification. Highly saline and anaerobic solution like those of the studied reservoir (supplementary material, Table S1) can then be a potential reservoir for ancient DNA. Clays coating the grains could also favour the adsorption of DNA, thus preserving it from hydration. Interestingly, archaeal isolates from Eocene rock salt were polyploid, with genome copy numbers of 11–14 genomes during exponential growth phase. Abundance of DNA in single archaeal cells in Rabi paleoenvironment could explain preservation of archaeal DNA. This strong hypothesis should get more confirmation.

With regard to the second hypothesis, dormant or slow activity communities have been recovered from tens of million years in a freshwater sediment or from a surface isolated lake in Antarctica. Moreover, microbial survival related to fermentative activity rather than sporulation has been proven on an up to a half a million years period due to fermentation processes in permafrost environment. Both metabolic and phenotypic features should be taken into consideration to explain the presence of microorganisms retrieved by cultural and molecular methods in the reservoir (e.g. sporeformers of the order Bacillales). As fermentative Prokaryotes constitute the large majority of the microorganisms detected by each technique, and given the amount of reduced hydrocarbon present in the Rabi reservoir, this hypothesis can be viewed as probable as the first. The most important question in the case of the studied oil reservoir is whether the retrieved archaeal 16S rRNA genes belong to dormant Archaea that are part of the non-cultivated microorganisms, or are part to dead microorganisms which have no more chance to be cultivated. Presence of both possibly active microorganisms and microorganisms inherited from the time of the sediment deposition suggests that the microbial diversity integrates separate parts of the reservoir history.

Studies of microbial diversity from a core rather from production fluids (e.g. formation water) in oil fields are rare and may not be accurate for comparison with our study, due to the difference in salinity. However, there are few reports on the predominance of aerobic microorganisms in oil reservoirs that are generally inhabited by strict anaerobes including sulfate-reducers and methanogens. The reservoir rock studied here belongs to a cretaceous formation dominated by evaporitic conditions. Protective presence of salt, adsorption on clays and low water activity could have preserved macromolecules such as DNA and may therefore explain the repeated molecular detection of aerobic hyperhalophilic Archaea. In this respect, we believe that molecular analyzes in particular provide a frozen picture of the past microbial community existing in the saline sedimentary basin, which is no more active in the hypersaline reservoir studied. This hypothesis is strengthened by the presence of archaean DNA sequences that are phylogenetically related to those retrieved from actual hypersaline ecosystems, but also from cultivated halooarchaea originating from similar extreme environments. In addition, the anaerobic halophilic bacterium that we have isolated pertains to the family Halobacteroidaceae (e.g. Halobacteroides spp.), which are common inhabitants of terrestrial saline ecosystems. In contrast, there are several examples of isolation from oil reservoirs in literature of bacteria pertaining to the family Halanaerobiaceae (e.g. Halanaerobium genus), which have been retrieved only by molecular approaches during the course of this study. In this respect, the novel isolated halophilic anaerobe might have been a microbial remnant of the original microbial community in the sedimentary basin. Finally, we demonstrate here that studying the microbiology of deep subsurface cores may be of geobiological significance by delivering important information on the existing microbial diversity at surface several millions years ago.

Methods

Core handling. A core was sampled within aluminum sleeves at 1153–1554 m depth below surface and immediately frozen (−80 °C) on the rigsite with liquid nitrogen. Reservoir pressure and temperature were respectively 12 Mpa and 43 °C. The core was defrosted in an anaerobic box glove chamber to ensure that no oxygen could impede development of anaerobic microorganisms. The chamber was decontaminated (bactericide and ethanol) and only sterilized materials or materials cleaned with a bactericide were introduced in order to preserve the core from any contamination during the sub-samples preparation. Only the inner part of the core (excluding ca. 2 cm in a 10 cm diameter core) was analyzed to avoid contamination by the drilling fluid. The full preservation of such unconsolidated sandstone witnesses the lack of drilling fluid invasion in the core.
Petrographical and chemical analyses. Samples were observed under binocular for macroscopic observation, and with SEM (Philips) for ultramicroscopic analysis. X-ray diffraction was performed on a Philips 0–20 (PW1050/81, PW3710) diffractometer. Clays were separated from the bulk sample by ultrasonication after hydrogen peroxide oxidation and grains (quartz–feldspar) sedimentation.

Porosity and permeability of the sandstone were respectively between 24% and 32% and 1 to 3.5 Darcy. The water content was 12.9%. The fluid had an alkaline pH value of 8.8. Organic carbon, measured on a Flash EA CHNS/O analyzer (Thermo-scientific), was 0.38%. Cations (Ca²⁺, Na⁺, K⁺, Mg²⁺) and metals (Fe, Mn, Co, Cu, Pb, Zn) were measured by ICP-AES, Jobin Yvon JY2000 Ultrace after mineralization of the sample. Anions (Cl⁻, NO₃⁻, SO₄²⁻) were determined by ion chromatography Dionex DX100. Phosphorus was measured by the Joret-Hébert method (NF X31 161), which consists of an extraction of phosphorus with oxalate before optical reading at 825 nm. Chemical analyses on the core material are given in the supplementary material Table S1.

DNA extraction. A portion of the sediment was washed with the aim to remove hydrocarbons and PCR inhibitory material and improve DNA recovery and efficiency of PCR amplification62. Washing was carried out by re-suspension in wash solution (2 mL per g of sample), vortexing for 2 min, followed by centrifugation at full speed for 5 min in a bench top microfuge (14,000 g). Three successive washes were performed in wash solution 1 (50 mM Tris–HCl pH 8.3, 200 mM NaCl, 5 mM Na₂EDTA, 0.05% Triton X-100), then in wash solution 2 (50 mM Tris–HCl pH 8.3, 200 mM NaCl, 5 mM Na₂EDTA), and finally in wash solution 3 (50 mM Tris–HCl pH 8.3, 0.1 mM Na₂EDTA). Despite there can be some potential loss of cell during these washing steps, this procedure was performed as it provided greater DNA quantities (data not shown). Bacterial genomic DNA was extracted from the washed sediment with the Fast DNA Spin Kit for Soil (Bio101) and the UltraClean Mega Soil DNA isolation kit (MoBio). The manufacturers protocols were slightly modified: as samples contained a priori low biomass, Poly-dIdC (polydeoxyinosinic–deoxyctydilic acid, Sigma), a synthetic nucleotide acting both as a blocking- and carrier-agent was added at the first step of the DNA extraction procedure63. DNA extracts were pooled and concentrated for subsequent PCR experiments.

For identifying members of the Bacteria domain, 16S rRNA genes were amplified with bacterial primer 8F (5′-AGAGTTTGTATCMTGGCTCAG-3′) and universal primer 1406R (5′-GACGGGCGGTGTGTRCA-3′), 30 cycles and hybridization at 55 °C. For identifying Archaea members, domain specific primers Arch363F (5′-ACGGGGYGCAGCAAGCGCA-3′) and Arch915R (5′-GTGCTCCCCCGCAAATTCCT-3′), 35 cycles and hybridization at 65 °C were used.

Clone libraries generation and screening. 16S rRNA gene libraries were constructed from gel-purified (Nucleospin extract II, Macherey-Nagel) PCR products using the TOPO-TA Cloning Kit for Sequencing (Invitrogen). The gene libraries were screened for correct-length inserts by direct PCR amplification from a colony using primers T3 and T7 targeting the plasmid. The V3 region of the insert of 926MiSeq.R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTATGGGAACTCTATCC-3′) and 926MiSeq.F (5′-GTGCTCAGGTAATGTTCAGGTTGTGTG-3′) for 20 cycles and hybridization at 52 °C. This step was then followed by a second PCR round using the universal primer pair 515F (5′-GTGNCAGCMGCCGCGGTA-3′) and 926R (5′-CCGYCAATTTYMTTTRAGTTT-3′) for 20 cycles and hybridization at 55 °C. The produced 440 bp long amplicons were then subjected to a third PCR amplification round using the 515MiSeq.F (5′-TCGTCCCGCCGACGTGCTGATATCC-3′) and 926MiSeq.R (5′-GTCTCGTGGGCTCGGAGATGTGTG-3′) primer pair which introduced Illumina’s overhang adaptors. Using Illumina’s Nextera Index kit, a final round of PCR amplification was then performed to introduce the indexes and remaining part of the Illumina’s adaptors according to the manufacturer’s recommendations.

The produced amplicons were cleaned up using the AMPure XP beads (Agencourt) and quantified on a Qubit fluorometer (Life Technologies) prior to pooling and sequencing on a MiSeq instrument (Illumina).

Sequence analyses. Sequence manipulations, analyses and alignments were performed using the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Similarities of concensus 16S rRNA gene nucleotides were determined using the BLASTN program (http://www.ncbi.nlm.nih.gov/BLAST).

Amplicon preparation for next-generation sequencing. Amplicons of the 16S rRNA gene suitable for sequencing on a MiSeq (Illumina) v3 kit (2 × 300bp) were produced using a nested PCR approach. During the first PCR reaction the nearly full length 16S RNA gene was amplified with the bacterial primers 8F (5′-AGAGTTTGTATCMTGGCTCAG-3′) and 1492R (5′-TACCTTGTAYGACTT-3′) for 20 cycles and hybridization at 52 °C. This step was then followed by a second PCR round using the universal primer pair 515F (5′-GTGNCAGCMGCCGCGGTA-3′) and 926R (5′-CCGYCAATTTYMTTTRAGTTT-3′) for 20 cycles and hybridization at 55 °C. The produced 440 bp long amplicons were then subjected to a third PCR amplification round using the 515MiSeq.F (5′-TCGTCCCGCCGACGTGCTGATATCC-3′) and 926MiSeq.R (5′-GTCTCGTGGGCTCGGAGATGTGTG-3′) primer pair which introduced Illumina’s overhang adaptors. Using Illumina’s Nextera Index kit, a final round of PCR amplification was then performed to introduce the indexes and remaining part of the Illumina’s adaptors according to the manufacturer’s recommendations.

The produced amplicons were cleaned up using the AMPure XP beads (Agencourt) and quantified on a Qubit fluorometer (Life Technologies) prior to pooling and sequencing on a MiSeq instrument (Illumina).
nitrate reducers; TMA (trimethylamine) or methanol for isolating methylo trophic methanogens. Media were also designed to isolate anaerobic sulfide-oxidizers and aerobic thiosulfate oxidizers. With the first results of molecular diversity becoming available, aerobic enrichments from core samples were also performed for isolation of aerobic and anaerobic halophilic microorganisms (see supplementary Table S5–S7). Media sterilized by filtration, and not autoclaving were also used, to prevent Maillard reaction that could impede prokaryotic development (see supplementary Table S6). pH was fixed at 8.8 (pH measured from the sediment), but 7.5, a close neutral value, was also tested, as both alkaline pH and highly saline conditions may limit growth and even survival of anaerobic micro-organisms. Temperature was fixed at 43 °C (in situ temperature).

Exclusion of contamination. Several criteria can be used to evaluate if an isolate or a 16S rRNA gene sequence can be considered as native to the formation of interest or not. This includes the sampling technique as discussed above. For instance, production waters collected from separators and wellhead samples have a higher probability to contain exogenous contamination than core samples6. Coring was performed prior to any fluid injection in the oil field, which prevented contamination by any anthropogenic fluid. Specifically designed techniques for coring in soft materials and adapted to integrity preservation were used (aluminum sleeves, adequate drilling parameters and fluid pressure). Moreover, freezing of the core on the rigsite in liquid nitrogen will have prevented further chance of chemical and microbiological contamination. Microscopic and SEM observations of the core did not show any physical evidence (disruption, mineralogical contamination) of penetration of the drilling fluid into the core. As the clays from core samples were green and not oxidized, it seems that coring processes did not affect physico-chemical conditions of the reservoir. Only the inner part (6 cm) of the core (10 cm) was inoculated into culture media or submitted to DNA extraction. Community structures (molecular CE-SSCP fingerprints of 16S rRNA genes, data not shown) of inner and outer (2–3 cm) compartments were highly similar, supporting the absence of detectable exogenous microbe contamination by the drilling fluid. Furthermore, microorganisms retrieved from the molecular surveys were related to physiological features compatible to in situ conditions. Another possibility to evaluate the contamination risk is to compare physiological adaptation of isolated microorganisms to the in situ physico-chemical conditions. The optimum temperature for growth of a microorganism isolated from an oil reservoir can be a good indicator of its physiological adaptation to the environment if it corresponds to the in situ temperature. In high saline reservoirs, comparison of salt tolerance or dependance of isolates can be compared to in situ water salinity as well. Culture optimal conditions (temperature 25–47 °C, NaCl 10–26%) of a strain isolated from the sediment and closely affiliated to the Halobacteroides genus were representative of the in situ conditions. All the halophilic microorganisms that we have isolated require subsequent concentrations of NaCl for growing, lowering the probability of microbial contamination during sample handling.

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51.第一批列表。
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Author Contributions
C.J. and S.C. conducted the molecular analyses with support from G.G. and I.N. High throughput sequence analyses were performed by N.T. Under the supervision of B.O., D.A., G.G. and I.N performed the isolation of the strains. G.G. and J.B. conducted the petrophysical observations. B.L. provided the sample and data from the studied oil field. G.G., C.J. and B.O. wrote the manuscript with input from all of the authors. C.J., G.G., D.M. and J.B. initially built the research project financed by SHELL Company.

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