Buried hydrocarbon deposits, such as liquid petroleum, represent an abundant source of reduced carbon for microbes. It is not surprising therefore that many organisms have adapted to an oily, anaerobic life deep underground, often at high temperatures and pressures, and that those organisms have had, and in some cases continue to have, an effect on the quality and recovery of the earth’s diminishing petroleum resources. There are three key microbial processes of interest to petroleum producers: reservoir souring, hydrocarbon degradation and microbially enhanced oil recovery (MEOR).

Reservoir souring
During reservoir souring, sulphate ions present in the formation of the added water are converted to hydrogen sulphide by the action of sulphate reducing bacteria (SRB). This represents a major challenge, in particular to offshore petroleum production, because of the concentration of sulphate in seawater and because H₂S is highly corrosive of the steel infrastructure. The incidence of souring following water flooding is a well-recognised phenomenon, and its economic impact is significant 1, 2.

Hydrocarbon degradation
Hydrocarbon degradation is the process in which the predominantly short- and medium-chain alkanes are metabolised, leaving heavy oil composed of long chains, aromatics and asphaltenes. This process is typically observed in reservoirs with a history of moderate temperatures. A commonly held belief is that temperatures above about 80°C prevent this degradation, presumably implying that the organisms responsible do not tolerate temperatures above 80°C 3. Despite that, hyperthermophilic microorganisms with optimal growth temperature above 80°C have been isolated from petroleum reservoirs 4, 5. Those thermophiles were mainly isolated from water-flooded reservoirs; however, L’Haridon et al. 6 have provided evidence that thermophilic bacteria and hyperthermophilic archaea exist in a non-flooded petroleum reservoir about 1670 metres deep and at a temperature of 65-70°C.

Aerobic degradation of petroleum hydrocarbons occurs rapidly at the surface, whereas anaerobic biodegradation occurs in the deep subsurface and is very slow 8, 9. A large number of bacteria, including the α-, β-, γ- and δ-subclasses of proteobacteria, have been shown to be able to metabolise various hydrocarbons under anaerobic conditions 10. Therefore further identification of bacterial distribution in reservoirs may provide more insight on the hydrocarbon biodegradation of deep-well oil fields.

Microbially enhanced oil recovery
The phrase microbially enhanced oil recovery (MEOR) encompasses a number of processes which increase the rate or extent of recovery of oil from a reservoir 11. As the easily recovered oil is recovered, producers are looking to recover more difficult to extract oil and MEOR is increasingly being considered as an option. In general terms, microorganisms, indigenous or introduced, are encouraged to grow, and in doing so produce gases, solvents, surfactants, etc, which might enhance oil recovery by a variety of mechanisms. These include a reduction in the viscosity of the oil, alteration of oil/rock interaction, selective plugging of porous zones and increasing pressure within the reservoir by gas production and other mechanisms.

Although numerous field trials have been performed (with varying degrees of success) 2, MEOR is far from predictable; the factors that govern the success of the process are not understood, and many questions remain. In addition to the engineering and geochemical issues, the identification of reservoir microbial diversity and its metabolic potential remain to be understood and controlled.
Central to the development of a reliable MEOR protocol will be an understanding of how the largely anaerobic microflora that exist in the reservoir respond to any stimuli that can be implemented from the surface. A key step is therefore to gain an understanding of the microbial diversity that is present in a particular reservoir. Traditionally, culture-based methods have been used as the primary means of bacterial identification and enumeration in oil fields. Culturing of bacteria from environmental samples is at best highly selective, and the problem is exacerbated when the environment is as extreme as that found in a deep petroleum reservoir. Therefore molecular techniques such as PCR \cite{2,13}, DGGE \cite{14} and FISH \cite{15} are now more widely used to probe environmental samples prior to addressing the activity and physiology and for assessing their activity and physiology. Here we present some preliminary results of a pilot study of the prokaryotic diversity present in an Australian petroleum reservoir.

Formation water was obtained from the operator of an Australian off-shore oil field. The sample was obtained from a depth of 1300m and a temperature of about 73°C. Two mL of formation water and 5mL ASL (Qiagen) buffer were mixed and incubated in 65°C for 1 hour, then a 1.6mL aliquot was removed and to it 2g Zirconia beads were added, and the suspension shaken in a bead-mill for 2 min at 25Hz. Then 1.2mL of supernatant was removed and microbial DNA was extracted using a QIAamp DNA Stool Mini Kit (Qiagen).

PCR amplifications were performed using bacterial- (16S U1 and 16S U2) \cite{16} and archaeal-selective (A751F and uA1406R) primers complementary to the genes encoding 16S ribosomal RNA (rRNA) \cite{17}. The amplicons were cloned and a small number selected at random for sequencing. The phylogenetic relationship between the clones is shown in Figure 1.\cite{18}

Eight clones derived from the bacterial selective primers were examined, and all were most similar (99-100%) to various species of Marinobacter including *M. hydrocarbonclasticus* and *M. vinifirmus*. *M. hydrocarbonclasticus* is an aerobic marine bacterium capable of degrading alkanes \cite{19}. Fourteen clones were sequenced at random from the population of archaeal-selective PCR amplicons. A single archaeon-related sequence was observed: a thermophilic methanogen, *Methanothermococcus*, which has previously been identified in oil reservoirs \cite{6,7,20}.

Unexpectedly, the remainder of the clones were related to bacterial 16S rDNA sequences. Some of those bacteria have properties consistent with survival in petroleum reservoirs, for example, *Thermoanaerobacter* and *Desulfatomaculum* are both thermophilic anaerobes observed previously in high temperature reservoirs \cite{6,7,21}. Two observations each were made of amplicons related to *Flavobacterium* and *Halomonas*, both of which are commonly observed in seawater, and are not unexpected in an offshore reservoir into which seawater has been injected. However, a large number of the clones were most

![Figure 1. Phylogenetic tree of clones observed in formation water. Nucleotide sequences were aligned with ClustalW and phylogenetic trees were created with MEGA 3.1 software using maximum parsimony. The significance of the junctions was established using the bootstrap method (1000 replicates) \cite{18} and junctions with a greater than 50% significance are labelled. MB1-1 to MB1-8 are clones obtained using bacterial primers (red discs) and MA1-1 to MA1-16 (black discs) are clones obtained with archaeal primers.](image)
closely related to *Phyllobacterium* sp., a genus of the order Rhizobiales, whose members are typically associated with plant roots and soil rather than oil reservoirs.

Although not presented here, DNA was extracted from 5mL of an oil sample from the same reservoir by bead beating followed by water extraction and method described above. The DNA obtained by that method was too dilute and too poor in quality to yield reliable PCR products.

This pilot study has demonstrated several points. The protocol generated a range of DNA clones, many of which are consistent with organisms which were expected in the reservoir. However, some of the clones were homologous to rRNA genes from organisms which would not be expected in the reservoir, therefore particular attention must be paid to the sampling and DNA extraction procedures to eliminate the possibility of contamination with non-reservoir organisms. Large volumes of water and oil will be processed in order to generate DNA of sufficient quantity and quality to detect microbes that may be present at very low levels in that phase. The archaeal-selective primer set used in this study amplified primarily bacterial sequences, so alternate primers should be investigated.

Finally, although studies such as this can yield important information about the prokaryotic diversity in the reservoir, new technology means that it is now possible to obtain gigabases of DNA sequence rapidly and cheaply, from which diversity information can be obtained – information which is unbiased by PCR primer design. Even more importantly, this approach (called ‘metagenomics’) \(^2\) would yield sequence information from not just the rRNA genes but from the bulk of the genome in each organism. Such information would tell us about the presence or absence of important functional genes and pathways, for example the pathways for the production of surfactants which, upon appropriate stimulation, might increase the flow of oil from the reservoir.

### References


Philip Hendry is a Principle Research Scientist in the Division of Molecular and Health Technologies at CSIRO. His research interests lie in the chemistry/biology interface and range from catalytic RNA to industrial applications of microbiology.

Dongmei Li is a Postdoctoral Fellow in the Division of Molecular and Health Technologies at CSIRO. Her research interests are in molecular biology and microbiology and their applications in identification of industrial microorganisms.