*International Journal of Astrobiology* 7 (2): 169–176 (2008) Printed in the United Kingdom
doi:10.1017/S1473550408004163  

# The use of complex microbial soil communities in Mars simulation experiments

Kai Finster[1], Aviaja A. Hansen[1], Lars Liengaard[1], Karina Mikkelsen[1], Tommy Kristoffersen[1], Jonathan Merrison[2], Per Nørnberg[3] and Bente Aa. Lomstein[1]

[1]*Department of Biological Sciences – Microbiology, University of Aarhus, 8000 Aarhus C, Denmark*
*e-mail: kai.finster@biology.au.dk*
[2]*Department of Physics and Astronomy, University of Aarhus, 8000 Aarhus C, Denmark*
[3]*Department of Earth Sciences, University of Aarhus, 8000 Aarhus C, Denmark*

**Abstract**: Mars simulation studies have in the past mainly investigated the effect of the simulation conditions such as UV radiation, low pressure and temperature on pure cultures and much has been learnt about the survival potential of sporeformers such as *Bacillus subtilis*. However, this approach has limitations as the studies only investigate the properties of a very limited number of microorganisms. In this paper we propose that Mars simulations should be carried out with complex microbial communities of Martian analogues such as permafrost or the deep biosphere. We also propose that samples from these environments should be studied by a number of complementary methods and claim that these methods in combination can provide a comprehensive picture of how imposed Martian conditions affect the microbial community and in particular the survival of its constituents – microbes as well as biological material in general. As an interesting consequence this approach can lead to the isolation of bacteria, which are more recalcitrant to the imposed Martian conditions than the pure cultures that have previously been studied.

*Received 29 May 2008, accepted 4 June 2008*

**Key words**: Extremophiles, permafrost, polyphasic approach, survival.

## Introduction

Ongoing Rover missions in combination with orbiting satellites have generated impressive quantities of extremely valuable data on the geological, physical and chemical conditions prevailing on our planetary neighbour, Mars (e.g. Horneck 2000; *Science* vol. 306). Consequently, the surface of Mars is now known in much greater detail than the surface of the Earth. It was a major scientific breakthrough when the presence of liquid water on the surface of Mars in its past was unambiguously demonstrated (Boynton *et al*. 2002; Krasnopolsky *et al*. 2004; Kuzmin *et al*. 2004). Water is considered a prerequisite for life. However, the presence of water is not synonymous with the presence of life. Thus, missions dedicated to clarifying questions related directly to the presence of recent or past life on Mars are required. These missions demand very sensitive analytical tools for the detection of living organisms (most likely microbes) because they may only be present in very low number. These tools, which will need to be operated remotely under Martian conditions, have yet to be developed. In the meantime astrobiological research, the search for life in the Universe, is dependent on terrestrial material that has physical, geological or chemical properties in common with Martian environments. NASA recently released a Roadmap, which canonizes a number of astrobiological research goals and has endeavoured to set up a framework of objectives for this diverse field of research (Des Marais *et al*. 2001). Herein, microbiology plays a central role. Overall, the terrestrial habitats that are studied have to be extreme, which in the Martian context stands for very dry, very cold and very nutrient poor and, due to the very low content of free oxygen in the atmosphere, anoxia should also be included. In addition, they should receive large fluxes of potentially lethal radiation such as UV light. However, UV radiation is only of importance when surface environments are in focus.

Samples that fit with all or some of the criteria are called 'analogues'. In the case of Mars the criteria for being an acceptable analogue are according to NASA met by a number of habitats such as cold and dry polar regions, permafrost soil, the deep biosphere, Rio Tinto and the Atacama desert. Studies of these environments may generate important information concerning the envelop of life on Earth. It may also be expected that ongoing research activities on Mars may provide information that forms the basis for a more extensive evaluation of the importance of those habitats that were

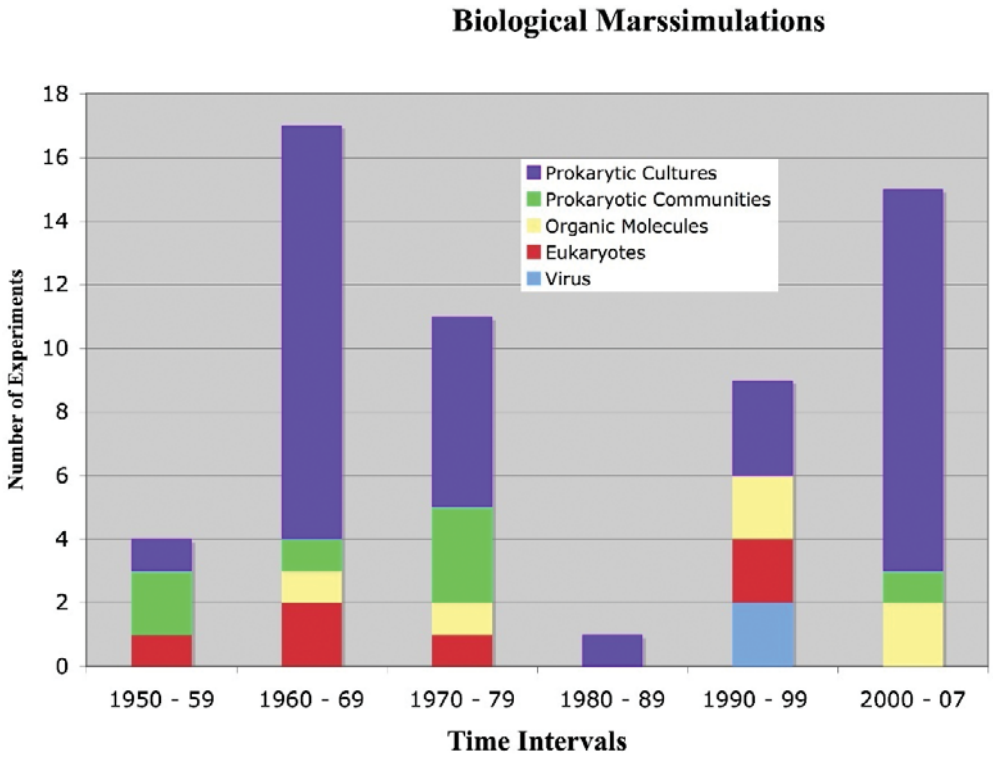


**Fig. 1.** Compilation of Mars simulation experiments that were carried out within the last 50 years.

hitherto regarded and studied as Martian analogues. Alternatively or in addition to studies of Martian analogues, we need to obtain answers to questions related to whether terrestrial microbes can survive on Mars, for how long and under what conditions? These questions are particularly pertinent in the context of planetary exploration and protection, since Martian microbes will need to be identified as unambiguously originating from the planet to be distinguished from terrestrial contaminants. These topics can all be addressed by Mars simulation experiments, an approach that has been used for almost 50 years (Fig. 1). Since the early days of Mars simulations much new background information has been obtained on the physical and chemical conditions present on Mars. These results have been incorporated into the design of simulation facilities to make these model systems more realistic. Furthermore, there have been major advances in the methodologies, which allow a more comprehensive evaluation of the effect of Martian conditions on terrestrial microbes.

In the present paper we evaluate and discuss the strengths and weaknesses of the suite of methods that may be employed to study the effect of short-term and long-term exposure of complex soil communities to simulated Martian conditions. The list of methods is not exclusive but forms the framework of our approach and may be extended by new upcoming methods and refinements in the future. We will also discuss the advantages of using complex microbial communities in simulation experiments compared to the hitherto used pure culture approach and conclude with a general discussion on the value of Mars simulations.

## Methods used to evaluate the effect of simulation conditions on the microbial community

A large and diverse toolbox of methods is available to investigate the structure, function and activity of natural microbial communities (Fig. 2), ranging from biogeochemical rate measurements to culture-independent molecular methods. They can be employed either individually or in combination to study the microbes and their activity in nature. We chose to combine as many methods as we have available to investigate the effect of a simulated Martian environment on the survival of terrestrial microbes (Hansen *et al.* 2005). In the following we will briefly describe the geometry of our experimental set-up and in more detail the methods that we have used in simulation experiments.

### *Preparation of samples*

Glass tubes were filled with freeze-dried, well-mixed soil samples and sealed from below with a Teflon stopper. The tubes were left open at the top thus exposing the soil surface to the UV light source. The heights of the soil cores were between 4.5 and 6 cm. Post exposure the cores were sliced into 1.5 to 20 mm thick horizons and subjected to the methods that we describe and discuss below. (A detailed description of our simulations facility can be depicted from our homepage (www.marslab.dk) or from a recently published simulator description (Jensen *et al.* 2008).)

### *Rate measurements with radioactive substrates*

The method is straightforward and very sensitive because radioactive carbon is not naturally present in the sample and

https://doi.org/10.1017/S1473550408004163 Published online by Cambridge University Press

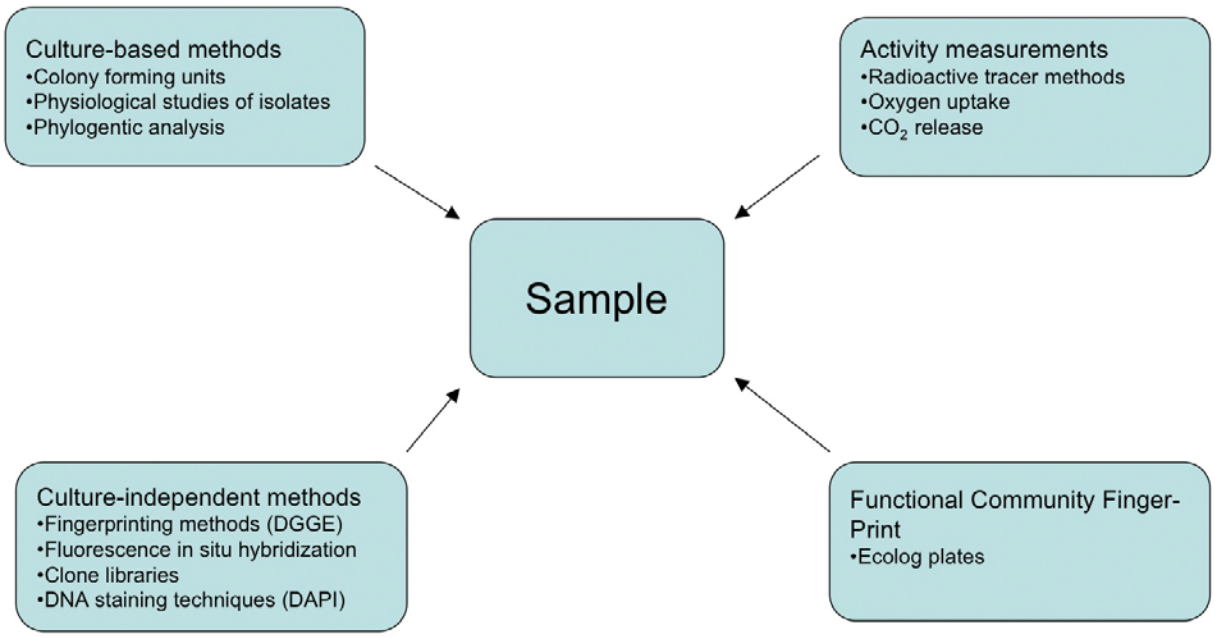


**Fig. 2.** Toolbox of methods for the evaluation of the effect of Mars simulation conditions on a microbial soil community.

can thus determine very low activities (Lund & Blackburn 1989). The method is recommended as a primary screening approach to test whether the simulation treatment had any effect on the metabolic potential of the microbial community in comparison to an untreated control. As the amount of material usually is limiting we scaled down the quantity of the material needed in the incubations, which can be carried out as follows (for details see Hansen *et al.* (2005)). 0.5 g of soil in glass containers is wetted with 0.5 ml physiological saline containing $^{14}$C-labelled substrate(s) (3 μCi μmol$^{-1}$, preferentially a cocktail of substrates including sugars, fatty acids and/ or amino acids) and aerated or ventilated with oxygen-free nitrogen if anoxia is required. The glass containers (five replicates for each horizon) are sealed with a rubber septum, incubated for up to 120 h in the dark at a constant temperature and stopped after appropriate time intervals by injection of 0.5 ml of a 2.5% NaOH (0.62 M) solution through the septum. The incubation time has to be determined in a pilot experiment and depends on the activity and the size of the indigenous microbial population in the sample. The amount of radioactive $CO_2$ produced as a function of respiratory activity is determined in a scintillation counter and the substrate oxidation rate constants of the different samples are calculated.

*Determination of the functional diversity from utilization patterns of a set of substrates* (*EcologTM*)

The substrate utilization pattern and thus the functional diversity of the soil community can be investigated using the EcologTM system (Fig. 3) (Biolog Inc., Hayward, CA). The system consists of a 96-well disposable plastic plate, which contains three identical sets of 31 freeze-dried substrates mixed with a tetrazolium salt and one substrate-free control (Masashi & Shoji 1999). The wells were inoculated with ~150 μl of a homogenized soil suspension that had been allowed to settle for about 10 min to remove the larger soil particles. Upon respiratory activity of the microbial soil community the colour of the well fluid turns red. Colour development can be evaluated by eye or read with a plate-reader. In the latter case the community activity can be expressed as average well colour development for the full plate and compared to plates inoculated with samples from the other soil horizons. In addition, the number of substrates can be compared among samples from the different horizons. The fact that each plate contains a triplicate set of substrates allows almost identical incubation conditions and the outcome from the different horizons can be analysed statistically using one-way ANOVA followed by Fisher's least significant difference test, when a significant effect was observed (Hansen *et al.* 2005).

A disadvantage of the Ecolog$^{TM}$ system is that only the aerobic or facultative anaerobe fraction of the microbial community can be evaluated because the colour only develops when oxygen is consumed during respiration. As a consequence obligate fermenting and obligate anaerobic microbes do not give a reaction. The soils that we studied required incubation times of the inoculated plates of up to one month. During the long incubation time care must be taken to avoid fungal contamination. If this happens the experiment has to be repeated. We were not able to obtained results with permafrost soil. No matter how carefully samples were prepared, the plates ended up being overgrown with fungi. No such problems were encountered when we used beech forest soil (Hansen *et al.* 2005). We have currently no explanation for these differences.

https://doi.org/10.1017/S1473550408004163 Published online by Cambridge University Press

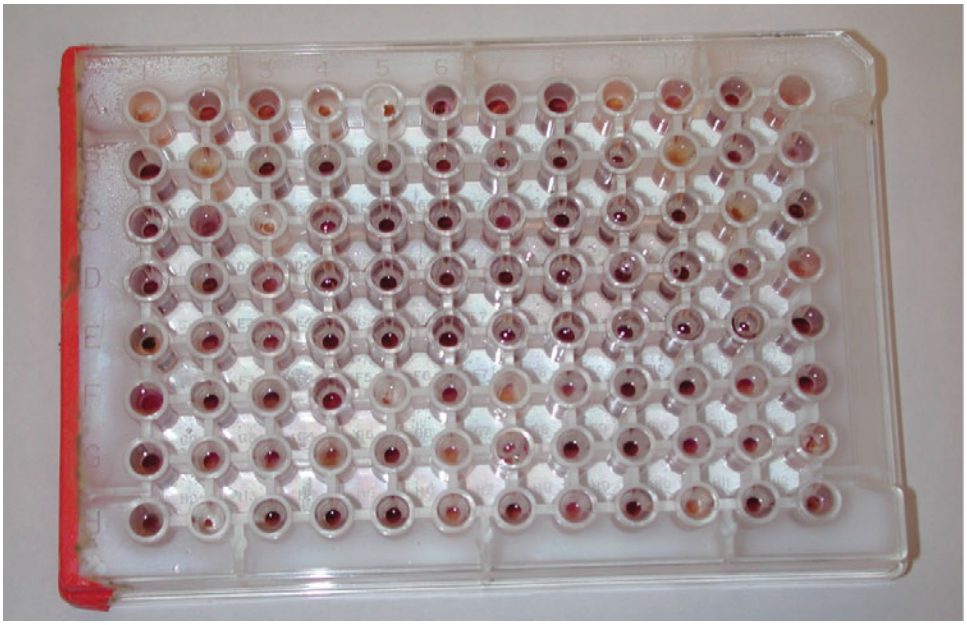

**Fig. 3.** Ecolog™ plate that was inoculated with iron-rich beech forest soil that was used as a Martian analogue. Soil microbes produce the red colour that developed in some wells by reduction of an added sodium tetrazolium salt.

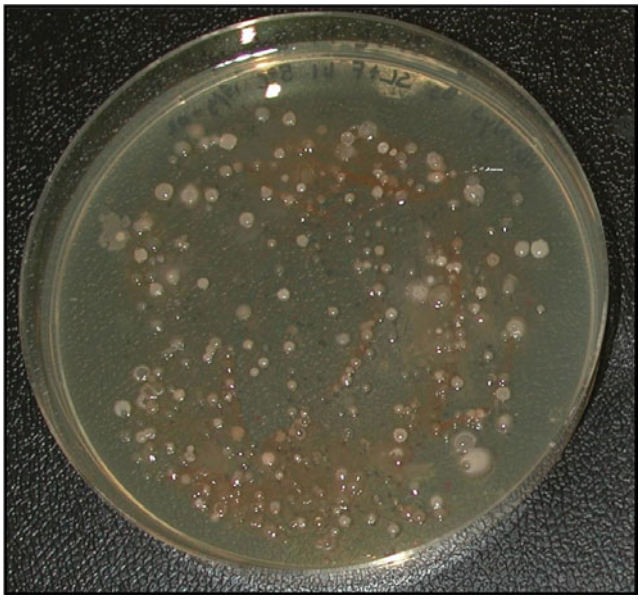

**Fig. 4.** R2A agar plate inoculated with a complex microbial soil community. The different colony morphologies are produced by different bacterial strains.

### *Viable counts or colony forming units*

The viable count method is a standard method used in microbiological investigations (Fig. 4). However, its validity has been questioned since the development of culture-independent molecular techniques (discussed below).

Usually an aliquot sample is suspended in an iso-osmotic saline solution and spread on nutrient agar plates. Since most Mars simulation experiments in the past have been carried out with pure cultures of aerobic bacteria the agar plate count technique of colony forming units has been used to evaluate the survival rate of the exposed culture. However, using soil instead of pure cultures, the evaluation is less reliable as the microbial community in the soil sample is complex and only a minor fraction of the total viable population will develop visible colonies on aerobically incubated nutrient agar plates. Since the introduction of culture-independent molecular methods in microbial ecology about two decades ago, culture-based methods have been widely criticized for only generating an incomplete and biased picture of the actual microbial community and for underestimating the size of the viable and active microbial community by several orders of magnitude (Olsen *et al.* 1994; Amann *et al.* 1995). Most often cited is a number given in Amann *et al.* (1995), stating that 1% of the *in situ* population at maximum can be cultured by conventional methods. Challenged by this criticism major efforts have been made in recent years to develop new media and to improve the incubation conditions in order to support growth of a larger fraction of the *in situ* community and thus bring 'ecologically relevant microbial players' into culture (Bruns *et al.* 2002; Kaeberlein *et al.* 2002; Sait *et al.* 2002; Gish *et al.* 2005). 'Relevant microbes' are defined here as microbes that are either numerically and/or functionally important for the processes that are occurring in the samples. Unfortunately, growth media cannot be applied generally and consequently media that were successfully used with one type of sample may not *per se* support growth of a large fraction of a microbial community in samples of another origin. Thus, it is recommended, although very time consuming, that different media are tested with the sample that has been selected for the simulation. We currently use R2A medium (Difco Laboratories, Michigan, USA), which has been designed to support the growth of microorganisms originating from nutrient poor habitats, such as the water column of oligotrophic lakes. Although R2A agar

https://doi.org/10.1017/S1473550408004163 Published online by Cambridge University Press

is nutrient rich compared to substrate concentrations found in many natural environments, R2A medium has much lower substrate concentrations than other commonly used growth media and thus may support the growth of a larger number of microorganisms than a nutrient rich medium (Zengler *et al*. 2002). R2A medium contains a broad spectrum of organic compounds and is thus potentially capable of supporting the growth of a diverse range of metabolically different microbes. However, despite the wide use of R2A medium new efforts continue to be made to develop media and techniques, which support the cultivation of a larger fraction of the bacteria in order to obtain a more comprehensive picture of the microbial community (Bruns *et al*. 2002; Kaeberlein *et al*. 2002; Sait *et al*. 2002; Zengler *et al*. 2002; Gish *et al*. 2005). Although 'classical' viable counts are time consuming and recover a small fraction of the viable microbial community they do, however, enable us to retrieve microorganisms with hitherto unknown physiological properties, e.g. properties that allow them to survive or even to be active under simulation conditions. However, if several different soil samples are to be tested prior to their use in a comprehensive Mars simulation experiment, one should consider whether viable counts are an essential part of this preliminary study.

Hitherto most simulation experiments, including our own, have focused on the effect of the simulation conditions on (facultative) aerobic microorganisms, primarily because they are easier to isolate and handle than anaerobes. However, given the low oxygen environment of present-day Mars and the parallel early low oxygen history of Earth following its formation, greater emphasis should be given to the anaerobes in future studies. In contrast to aerobic bacteria, the cultivation of anaerobic bacteria is more laborious and technically more difficult, and requires specialist equipment to remove oxygen from the samples. The proportion of anaerobic microbes can be determined by the most probable number methods in sealed anoxic test tubes or on agar plates inoculated and incubated under anoxic conditions in a glove box.

### *Molecular fingerprinting by denaturant gradient gel electrophoresis*

This method provides a relatively rapid procedure for determining the diversity of the major groups within a microbial community (Muyzer *et al*. 1993). It can be used to detect major changes of the structure of the microbial community. This pattern change may be the result of a perturbation such as the conditions imposed during a Mars simulation experiment. In order to be detectable the nature of the perturbation has to be such that it either eliminates or drastically decreases the amount of DNA present in the organisms, which are affected negatively by the imposed conditions. Simply killing the organisms without destroying their DNA would not change the outcome of the experiment. Denaturant gradient gel electrophoresis (DGGE) shares this weakness with all other DNA/RNA-based molecular techniques. In addition, the DGGE method only detects the members of a microbial community that represent at least 1–9% of the total microbial community. Recent studies have shown that this method should not be used when the microbial community is made up of a large number of small sub-populations. In this case stochastic events during PCR (Polymerase Chain Reaction) amplification of the extracted DNA result in a variety of patterns that do not reflect an effect of the perturbation, but are most likely an effect of random sampling (Gish *et al*. 2005). Briefly, the DGGE method makes use of differential melting of a mixture of PCR-amplified double-stranded sequence stretches of the 16S rDNA genes extracted from an environmental sample on a denaturant gradient agarose gel (Muyzer *et al*. 1993) (Fig. 5). The double-stranded 16S rDNA sequences are prevented from complete melting by the addition of short artificially high GC (Guanosine/Cytosine) tails. Melting by denaturant chemicals of the double-stranded DNA prevents the molecule from migrating further in the electric field through the agarose gel. The distinct bands formed by the melted 16S rDNA molecules are visible on the gel under UV light after staining with a RNA/DNA dye such as ethidium bromide. The band patterns, which are considered the fingerprint of the microbial community of the investigated samples, can be subjected to statistical analysis and the outcome of the analysis can be organized in dendograms (Hansen *et al*. 2005). For more detailed analysis the bands can be cut out of the gel with a scalpel. The containing DNA can then be extracted from the gel and further purified, amplified and sequenced. The partial 16S rDNA sequence, even relatively short pieces, may be compared to sequences in the databases, which reveals information on the phylogeny of the microorganism containing the sequence. This analysis, although time consuming, is very useful and provides a check of the comparability of the sequence patterns. It is has been widely assumed that sequences that migrate equal distances in the gel are identical. However, recent studies have challenged this assumption. In particular, communities with large species richness (many numerically small sub-populations) have been shown to contain strains with equal migration patterns although they were affiliated with different relatives. Consequently, careful analysis of the fingerprints by sequencing of selected bands is recommended. Similar precautions should be taken when using alternative fingerprinting methods such as t-RFLP (terminal restriction fragment length polymorphism).

### *Diversity studies using clone libraries*

Since cultivation-based methods for studies of the diversity of microbial populations in natural and man-made habitats have been widely criticized, because of their biases and incomplete coverage, alternative methods are needed. These methods are primarily based on sequence differences of the 16S rDNA gene among the strains that constitute the microbial population. One widely used molecular method is termed the 'clone library'. In an analogous manner to the previously described DGGE fingerprinting technique this method also depends on PCR amplification of 16S rDNA genes of DNA extracts from environmental samples and may thus meet similar biases.

https://doi.org/10.1017/S1473550408004163 Published online by Cambridge University Press

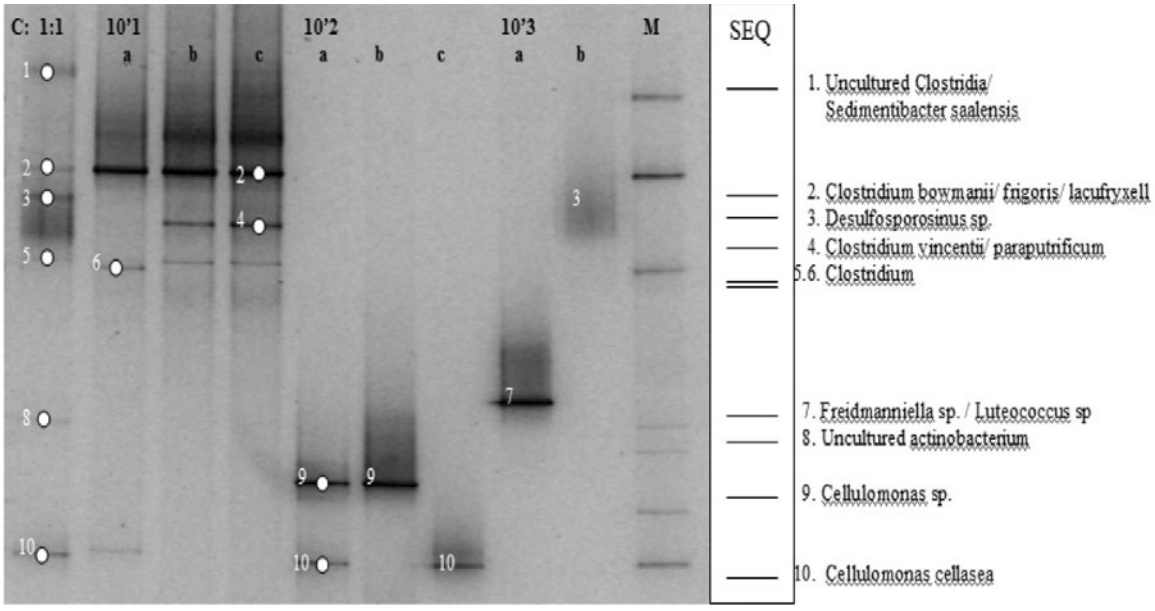


**Fig. 5.** Denaturant gradient gel electrophoresis (DGGE) gel. Different lanes show patterns of genetic diversity from different dilution steps. Each black band on the gel originates from an approximately 600 base pair long sequence of a 16 S rDNA gene. After separation on the gel selected bands were cut out and the sequences of the rDNA molecules were obtained. The names on the right-hand side of the gel are the names of the microbial strains whose sequences were most closely related to the sequences determined form material that was cut out of the gel. The closest relative was found by comparison of the obtained sequences with sequences stored in the GenBank database.

In principle, the method works as follows: DNA is extracted from a sample followed by PCR amplification of the pool of 16S rDNA genes using general primers. By transferring single 16S rDNA genes sequences from the mixture into competent *E. coli* cells 16S RNA genes are separated from each other. Successfully transformed *E. coli* cells form colonies on selective media. Single colonies are withdrawn from the agar plates and the taken-up 16S rDNA, now separated from the natural sample, is PCR amplified, cleaned, sequenced and compared to sequences in databases. In studies addressing the diversity of a natural sample a large number of clones needs to be analysed to exhaust the pool of different sequences in the mix. The number of clones that need to be sequenced to cover the genetic diversity of the microbial community inhabiting the sample is determined by statistical methods (Hansen *et al.* 2007). However, a careful study by Leser *et al.* (2002) of randomly complied sub-libraries of an impressive library of more than 4000 clones of 16S rDNA sequences showed that the different libraries reached saturation although made up of different representatives of the overall library. This result indicates that for samples that contain microbial populations that are made up of many small sub-populations large libraries are needed to evaluate the microbial diversity. It also demonstrates that statistical analysis of the compiling clones may result in inconsistent results. In addition, unavoidable PCR biases have to be considered. These biases may in the future been overcome by newly developed high throughput sequencing methods that are not dependent on a PCR step (Sogin *et al.* 2006). Overall clone libraries give a qualitative rather than a quantitative picture of the microbial community. Consequently PCR-based culture-independent methods produce exclusively qualitative results and are thus in many respects not more reliable than the culture-dependent methods. Still, culture-independent methods have drawn our attention to the tremendous microbial diversity present in natural environments, which has not been previously accessible using standard culture-dependent methods.

### *Fluorescence* in situ *hybridization*

Culture-independent methods where ribosomal RNA is stained with short (around 16 nucleotides), more or less specific, fluorescently labelled complementary sequences (FISH, fluorescence *in situ* hybridization) are useful to quantify the number of specific sub-populations present in the sample (Amann *et al.* 1995; Sekar *et al.* 2003). The probes allow detection ranging from the domain (Bacteria or Archaea) to the strain level. A significant drawback of the method is that it cannot distinguish metabolically active from dormant or even dead cells.

## Why study complex soil communities?

Mars simulation experiments that address the survival of terrestrial microbes under simulated Martian conditions may be carried out with either pure cultures or complex microbial communities. Both approaches have their advantages and disadvantages, which we will briefly discuss below. Pure culture studies allow the testing of the response of the selected bacterial strain to the simulation conditions. In most recent simulations strains belonging to the genus *Bacillus* were investigated (Jensen *et al.* 2008). These strains are aerobic

https://doi.org/10.1017/S1473550408004163 Published online by Cambridge University Press

soil bacteria, which produce heat, desiccation and radiation-resistant endospore. A detailed knowledge of the genetics and physiology of these strains allows very specific questions to be asked concerning the properties that enable them to survive the simulation conditions. The results that can be obtained from these investigations are particularly important when topics such as the 'the contamination of extraterrestrial bodies with terrestrial microbes' or 'the possibilities of terra-forming of Mars' are discussed (Graham 2004). For example, testing of spacecraft that were scheduled to land on Mars showed that they contained viable spore-forming bacteria, even after exposure to certified sterilization procedures (La Duc *et al*. 2003). Among the microbes that were isolated from the spacecraft were strains belonging to the genus *Bacillus* (Venkateswaran *et al*. 2004). The possibility of Man-induced Panspermia is a potential problem when searching for life in the Universe. A major drawback of pure culture studies is that we only obtain new information on the strains that we have picked as model organisms. However, many as yet undiscovered microbes might be present in the environment that may have alternative and even better survival strategies. These strains may be discovered and isolated in Mars simulation experiments using natural microbial communities, thus providing new insights into strategies that microbes have evolved to survive imposed extraterrestrial conditions. In addition, it is well-established that microbes integrate themselves into very complex webs of interaction, which are essential for their successful survival in their habitats. Thus using complex communities in their genuine growth matrix may provide more comprehensive insight into their response to the demanding simulation conditions.

## Methods and detection of life on Mars

The major driver behind the implementation of the different methods in astrobiological research in general and Mars simulations in particular as well as their improvement is the perspective of using them in the 'field', which in this case means Mars or other extraterrestrial bodies.

Despite inherent problems of culture-independent methods they are probably the most promising tools for life detection on Mars or elsewhere in the Universe because they do not depend on cultivation of the organism. However, there are a number of intrinsic assumptions associated with culture-independent methods that have to be made when searching for extraterrestrial life (problems extensively discussed in Pool & Willerslev (2007)). Firstly, in order to unambiguously identify extraterrestrial life we must assume that these putative life forms possess the same types of informational molecules as terrestrial organisms. If they do not use the same or similar information coding molecules (DNA, RNA) we will not be able to detect them using molecular methods. Secondly, all molecular identification methods depend on genes, which can be addressed by their conserved sequence regions serving as the targets either for primers in PCR-based techniques or probes in FISH studies. These regions need to be identified before the methods can be applied successfully. Thirdly, successful detection of living organisms requires a certain density of the organisms. Life on Mars may be so sparse that it may be well below the detection limits of the currently developed methods. The fact that there are surface regions on Earth, such as the Atacama desert, where living organisms cannot be detected either by culture-dependent and culture-independent methods is indicative of the formidable technical challenges involved in developing a successful life detection system on Mars (Navarro-Gonzáles *et al*. 2003). Finally, the methods also need to work ex-laboratorium under the environmental conditions prevailing in the extraterrestrial habitat under investigation. Considering the details and minor modifications that have to be made when the methods are used under controlled laboratory conditions, this represents a major challenge to the stability and automation of these methods. The first two points can only be addressed hypothetically because we cannot do anything about these if life on extraterrestrial bodies expresses itself differently from that with which we are familiar with on Earth. The last two points, however, present formidable methodological challenges to method developers. The improvement in the detection limit of not only the molecular methods but any of the above-addressed methods as well as their reliable automation would make a significant contribution not only to astrobiology but also to microbial ecology in general (Glavin *et al*. 2004; Sims *et al*. 2005).

Alternatively, microscopic methods could be applied. Here we assume, however, that complex, ordered structures may be living entities or their fossilized remains. We risk, however, identifying well-structured inorganic crystals as a biological structure (Monod 1971), if microscopy is the only method applied to the sample. A recent example of this type of potential pitfall was the interpretation of abiotic nano-bacteria-like structures in meteorite AHL84001 as signatures of fossilized Martian life (McKay *et al*. 1996). In combination with other methods such as Raman spectroscopy, however, which could be used to investigate the chemical makeup of the structure, microscopy could become a reliable and useful tool in future missions. Also with respect to these methods, and likewise molecular approaches, instrument development and testing under conditions as close to *in situ* as possible is essential for the reliability of results of future life-seeking missions to Mars or elsewhere in the Universe. As long as terrestrial specimens can be misinterpreted by the methods and abiotic chemical structures risk being identified as biological, any method is questionable and its application on missions should be considered with caution.

## Why Mars simulation experiments at all?

The most obvious and simple response to this question is that this is the best we can achieve at the moment since we do not have access to sample material from Mars. More seriously Mars simulation experiments are crucial for several reasons.

1. By simulation experiments we can address the effect of Martian conditions on microbes and learn from the outcome of these studies to what extent terrestrial

https://doi.org/10.1017/S1473550408004163 Published online by Cambridge University Press

microorganisms can tolerate/survive the imposed conditions. This is important, when we discuss the problems involved with sending landers to Mars and the potential risk of contaminating Mars with terrestrial microbes.
2. Mars simulation experiments may be used to isolate microbes that are particularly successful in coping with the conditions on Mars and thus may be used in future terraforming projects.
3. Finally, due to the detection limits of all methods, it is least as difficult to prove the absence of life as it is to prove its presence – thus method improvement is the mantra of astrobiological research and in this context simulation experiments are indispensable testing grounds.

Thus, Mars simulations may be used to improve and develop the methods to be used for the search for life during the forthcoming Mars missions. Considering the extremely high costs of space research it is vitally important to have very sensitive and reliable automated sets of methods available that can be used to detect living organisms, their activity and/or their remains even when present in very low numbers.

## Acknowledgements

The work was supported by FNU grant 272-06-0576. We thank Professor Rodney Herbert for a very careful revision of our manuscript. We also thank an anonymous reviewer for the critical comments that helped to improve the manuscript.

## References

Amann, R.I., Ludwig, W. & Schleifer, K.H. (1995). Phylogenetic identification and *in situ* detection of individual cells without cultivation. *Microbiol. Rev*. **59**, 143–169.

Boynton, W.V. *et al*. (2002). Distribution of hydrogen in the near surface of Mars: evidence for subsurface ice deposits. *Science* **297**, 81–85.

Bruns, A., Cypionka, H. & Overmann, J. (2002). Cyclic AMP and acyl homoserine lactones increase the cultivation efficiency of heterotrophic bacteria from the Centra Baltic Sea. *Appl. Environ. Microbiol*. **68**, 3978–3987.

Des Marais, D. *et al*. (2001). The NASA astrobiological roadmap. *Astrobiology* **3**, 211–235.

Gish, F., Schubert, K., Bruns, A., Hoffelner, H. & Overmann, J. (2005). Specific detection, isolation, and characterization of selected, previously uncultured members of the freshwater bacterioplankton community. *Appl. Environ. Microbiol*. **71**, 5908–5919.

Glavin, D.P., Cleaves, H.J., Schubert, M., Aubrey, A. & Bada, J. (2004). New method for estimating bacterial cell abundances in natural samples by use of sublimation. *Appl. Environ. Microbiol*. **70**, 5923–5928.

Graham, J.M. (2004). The biological terraforming of Mars: planetary ecosystems as ecological succession on a global scale. *Astrobiology* **4**, 168–195.

Hansen, A.A., Merrison, J., Nørnberg, P., Lomstein, B.Aa. & Finster, K. (2005). Activity and stability of a complex bacterial soil community under simulated Martian conditions. *Int. J. Astrobiol*. **4**, 135–144.

Hansen, A.A., Herbert, R.A., Mikkelsen, K., Jensen, L.L., Kristoffersen, T., Tiedje, J.M., Lomstein, B.Aa. & Finster, K.W. (2007). Viability, diversity and composition of the bacterial community in a high Arctic permafrost soil from Spitsbergen, Northern Norway. *Environ. Microbiol*. **9**, 2870–2884.

Horneck, G. (2000). The microbial world and the case of Mars. *Planet. Space Sci*. **48**, 1053–1063.

Jensen, L.L., Merrison, J., Hansen, A.A., Mikkelsen, K.A., Kristoffersen, T., Nørnberg, P., Lomstein, B.Aa. & Finster, K. (2008). A facility for long-term Mars simulation experiments. *Astrobiology* (in press).

Kaeberlein, T., Lewis, K. & Epstein, S.S. (2002). Isolating "uncultiviable" microorganisms in pure culture in a simulated natural environment. *Science* **296**, 1127–1229.

Krasnopolsky, V.A., Maillard, J.P. & Owen, T.C. (2004). Detection of methane in the Martian atmosphere: evidence for life? *Icarus* **172**, 537–547.

Kuzmin, R.O., Zabalueva, E.V., Mitrofanov, I.G., Litvak, M.L., Boynton, W.V. & Saunders, R.S. (2004). Regions of potential existence of free water (ice) in the near-surface Martian Ground: Results from the Mars Odyssey high-energy neutron detector (HEND). *Sol. Syst. Res*. **38**, 1–11.

La Duc, M.T., Nicholson, W.L., Cern, R. & Venkateswaran, K. (2003). Microbial characterization of the Mars Odyssey spacecraft and its encapsulation facility. *Environ. Microbiol*. **5**, 977–985.

Leser, T.D, Amenuvor, J.Z., Jensen, T.K., Lindecrona, R.H., Boye, M. & Møller, K. (2002) Culture-Independent Analysis of Gut Bacteria: the Pig Gastrointestinal Tract Microbiota Revisited. *Appl. Environ. Microbiol*. **68**, 673–690.

Lund, B.A. & Blackburn, T.H. (1989). Urea turnover in a coastal marine sediment measured by a $^{14}$C-urea short-term incubation. *J. Microbiol. Meth*. **9**, 297–308.

Masashi, G. & Shoji, T. (1999). A method of profiling microbial communities based on a Most-Probable-Number assay that uses BIOLOG plates and multiple sole carbon sources. *Appl. Environ. Microbiol*. **65**, 4419–4424.

McKay, D.S., Gibson, E.K. Jr., Thomas-Keprta, K.L., Vali, H., Romanek, C.S., Clemett, S.J., Chillier, X.D.F., Maechling, C.R. & Zare, R.N. (1996) Search for past life on Mars: possible relic biogenic activity in martian meteorite AHL8400. *Science* **273**, 924–930.

Monod, J. (1971). Le Hasard et la Necessite; essai sur la philosophie naturelle de la biologie moderne. Edité par SEUIL, Paris.

Muyzer, G.S., De Waal, E.C. & Uitterlinden, A.G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol*. **59**, 695–700.

Navarro-González, R. *et al*. (2003). Mars-like soils in the Atacama Desert, Chile, and the dry limit of microbial life. *Science* **302**, 1018–1021.

Olsen, G.J., Woese, C.R. & Overbeek, R. (1994). The winds of evolutionary change: breathing new life into microbiology. *J. Bacteriol*. **176**, 1–6.

Poole, A.M. & Willerslev, E. (2007). Can idenstification of a fourth domain of life be made from sequence data alone, and could it be done on Mars? *Astrobiology* **7**, 801–814.

Sait, M., Hugenholtz, P. & Janssen, P. (2002). Cultivation of globally-distributed soil bacteria from phylogenetic lineages previously only detected in cultivation-independent surveys. *Environ. Microbiol*. **4**, 654–666.

*Science* **306**, 1985–2148.

Sekar, R., Pernthaler, A., Pernthaler, J., Warnecke, F., Posch, T. & Amann, R. (2003). An improved protocol for quantification of freshwater *Actinobacteria* by fluorescence in situ hybridization. *Appl. Environ. Microbiol*. **69**, 2928–2935.

Sims, M.R., Cullen, D.C., Bannister, N.P., Grant, W.D., Henry, O., Jones, R., McKnight, D., Thompson, D.P. & Wilson, P.K. (2005) The specific molecular identification of life experiment (SMILE). *Planet. Space Sci*. **53**, 781–791.

Sogin, M.L., Morrison, H.G., Huber, J.A., Welch, D.M., Huse, S.M., Neal, P.R., Arrieta, J.M. & Herndl, G.J. (2006) Microbial diversity in the deep sea and the underexplore "rare biosphere". *Proc. Natl. Acad. Sci. USA* **103**, 12115–12120.

Venkateswaran, K., Chung, S., Allton, J. & Kern, R. (2004). Evaluation of various cleaning methods to remove *Bacillus* spores form spacecraft hardware materials. *Astrobiology* **4**, 377–390.

Zengler, K., Toledo, G., Rappé, M., Elkins, J., Mathur, E.J., Short, J.M. & Keller, M. (2002). Cultivating the uncultured. *Proc. Natl Acad. Sci. USA* **99**, 15 681–15 686.