



# Microbial biological indicators for the Mars Sample Return break-the-chain sterilization strategy

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The decision to implement Mars Sample Return will not be finalized until NASA's completion of the National Environmental Policy Act (NEPA) process. This document is being made available for information purposes only.  
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- Several on-orbit containment and sterilization modalities are currently being evaluated for potential Mars Sample Return (MRS) as break-the-chain (BTC) of contact strategies.
- An acceptable evaluation of the efficacy of BTC processes requires a thorough assessment of potential biological indicators (BI) to determine their suitability for inclusion in the applied approach.
- Thus, the objectives of this activity are threefold:
  1. To identify a set of BI's that could reasonably approximate the physiological and functional characteristics expected of Martian life, if it exists.
  2. To provide a recommendation on which BI's are most appropriate to use in BTC verification activities based on modality type.
  3. Once identified and approved, to evaluate the efficacy of various sterilization and containment modalities against the selected BI's.

# Introduction and Background

## Searching for a suitable biological indicator



### Phylogenetic Tree of Life

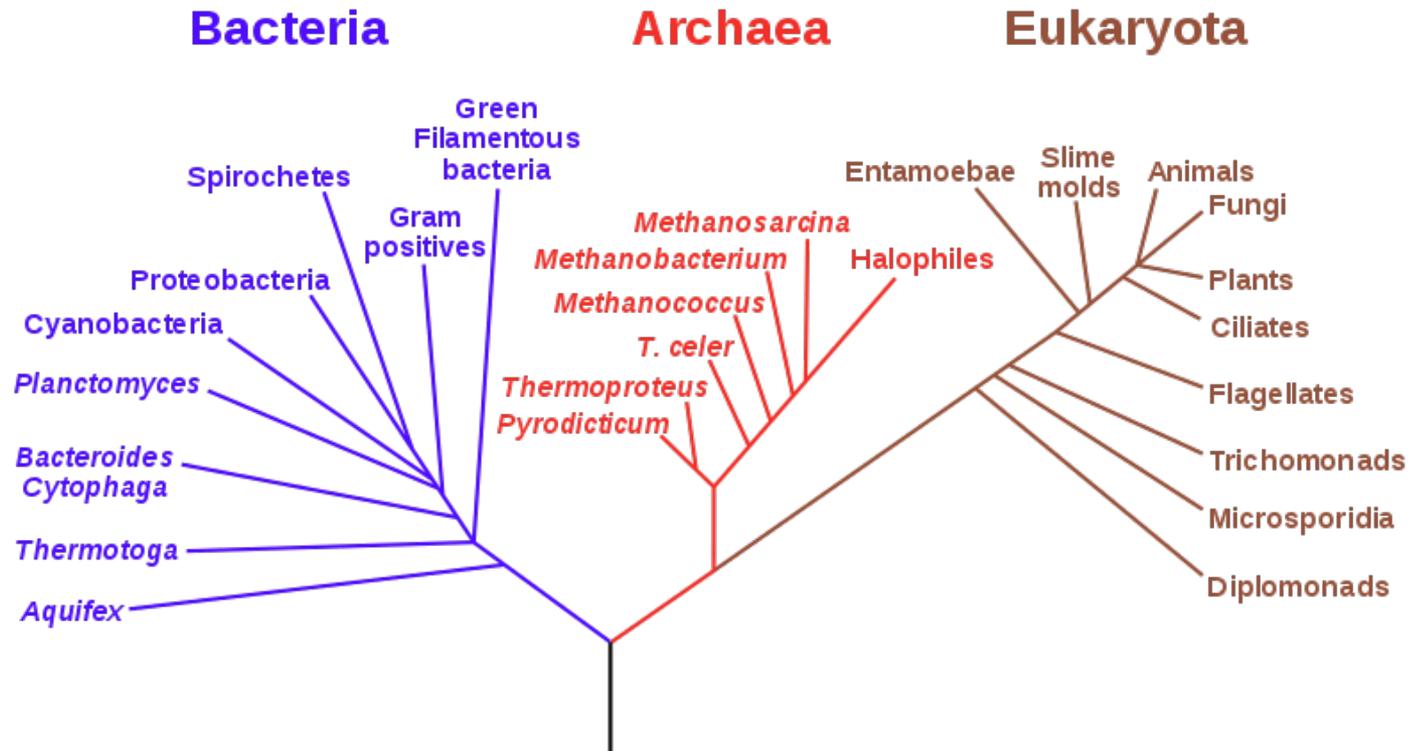


Image: Wiki commons/public domain.

# Potential candidates for BI consideration

## Archaea



- Archaea are non-endospore forming prokaryotes which occupy one of the three domains of life.
- Thought to be an ancient lineage, archaea share both morphological and genetic similarities organisms belonging to the Bacteria and Eukarya domains of life, but also exhibit novel characteristics as well.
- The archaea are considered “extremophiles” and exhibit many characteristics responsible for survival under harsh, early Earth primordial conditions which make it a potential candidate BI for MSR BTC sterilization strategies.
- Many species of archaea exhibit a remarkable ability to thrive in extremely inhospitable conditions. This ability can be attributed to physiological and/or genetic resistance mechanisms, as well as to their ability to metabolize a variety of energy sources which are often not suited for most other organisms.
- **Although an intriguing group to consider, Archaea exhibit several qualities which make it a less-than-ideal candidate for MSR BTC BI.**
  - Non-spore forming
  - Notoriously difficult to cultivate and isolate
  - Their use as BI’s has not been developed
  - Not widely accepted as a BI for sterilization processes



Acid mine archaea  
Courtesy: NASA

# Potential candidates for BI consideration

## Lichens



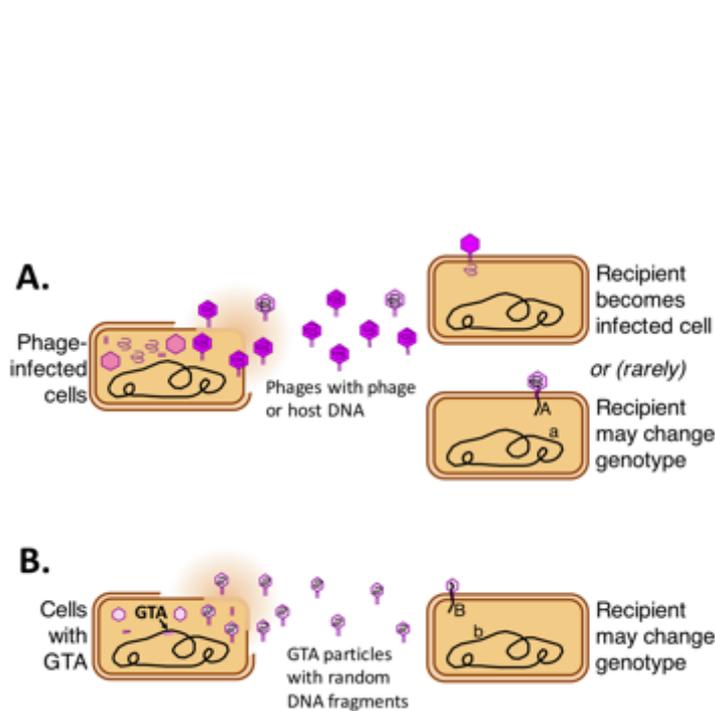
- Lichens are symbiotic organisms created by a fungal and photosynthetic partner with the capability to form even more complex associations.
- Some lichens, such as the Antarctic endemite lichen *Buellia frigida* were shown to survive exposure to low-Earth orbit–space and simulated Mars conditions (Meeßen et al., 2015).
- Lichens are tolerant of cold stress at long durations of time, and are known to be tolerant to heat 100° C when in the dry state, but their heat tolerance rapidly diminishes with increasing time of exposure.
- While lichens may be able to provide some useful information through research efforts centered on their known resistance mechanisms, based on the intersection of their large size and low environmental stress tolerance (relative to other candidates), **we do not consider lichens to be a suitable biological indicator candidate to verify a MSR BTC sterilization strategy.**



Lichen on rock.  
Photo: CC0/public domain

# Potential candidates for BI consideration

## Gene Transfer Agents



Comparison of typical phage (bacteriophage) infection and transduction (A) with typical GTA (gene transfer agent) production and transduction (B).

Image: Rosie Redfield

- Gene transfer agents (GTAs) are DNA-containing virus-like particles that are produced by some bacteria and archaea and mediate horizontal gene transfer.
- Along with bacteriophages, which can transfer genes and nucleic acids from one organism to another, GTA's can transfer transducing particles from one bacterial cell to another.
- GTA's have an average size of 30-80 nm.
- Since both viruses and GTAs require specific host for replication, the potential risk for contacting a Mars-borne GTA-like particle is far lower or negligible compared to free-living, self-replicating organisms, but still cannot be stated as zero.
- GTA's are highly susceptible outside their host, and can be easily inactivated using standard microbiology techniques.
- Due to other more suitable and easily manipulated candidates, **we do not consider GTA's to be a suitable biological indicator candidate to verify a MSR BTC sterilization strategy.**

# Potential candidates for BI consideration

## Nucleic Acids and other biogenic compounds



- DNA, RNA, and other nucleic acids are macromolecules that serve as the primary repository of genetic information in living organisms.
- Nucleic acids and other biogenic compounds are generally very susceptible to inactivation by physical, chemical, mechanical, and enzymatic processes.
- **Although they have had a foundational utility in a variety of MSR-related research efforts, nucleic acids do not appear to be suitable BI candidates for an MSR BTC sterilization strategy.**

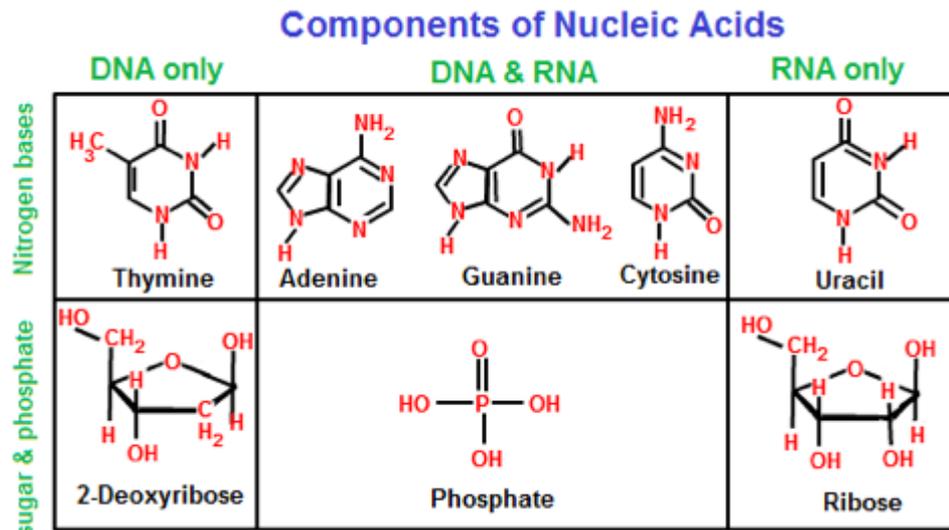


Image: <https://biology.tutorvista.com/biomolecules/nucleic-acids.html>

## Prions

- Prion proteins are complex proteinaceous molecules with a radius of approximately 10 nm and consist of 23 - 230 amino acids.
- Prion related diseases, also known as transmissible spongiform encephalopathies (TSE), can occur in a variety of mammalian hosts such as cattle, sheep, deer, domestic cats, and humans
- Prion inactivation requires denaturation of the protein into a non-functional form.
  - Inactivation can occur through protein hydrolysis or physical destruction of the molecule via heat-pressure treatments or chemical exposure.
  - The Centers for Disease Control and Prevention (CDC) recommended prion inactivation methods for instruments and surfaces to include combined heat and chemical treatment for prion inactivation.
- **There are a number of reasons which may exclude prions as a suitable candidate BI including:**
  - **Low resistance to environmental stress**
  - **Lack of research knowledge**
  - **Difficult to replicate, manipulate and handle**
  - **Increased risk as a potential health hazard**

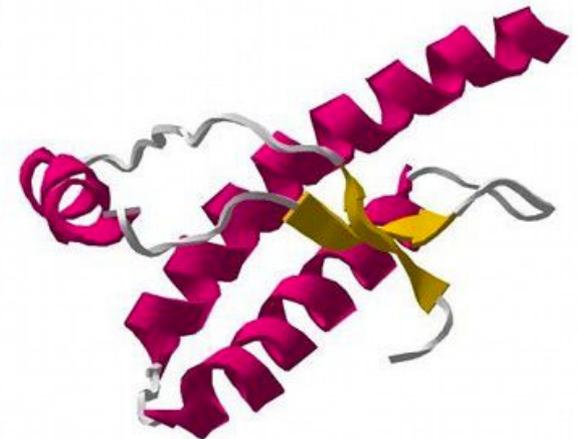


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# Potential candidates for BI consideration

## Bacterial endospores



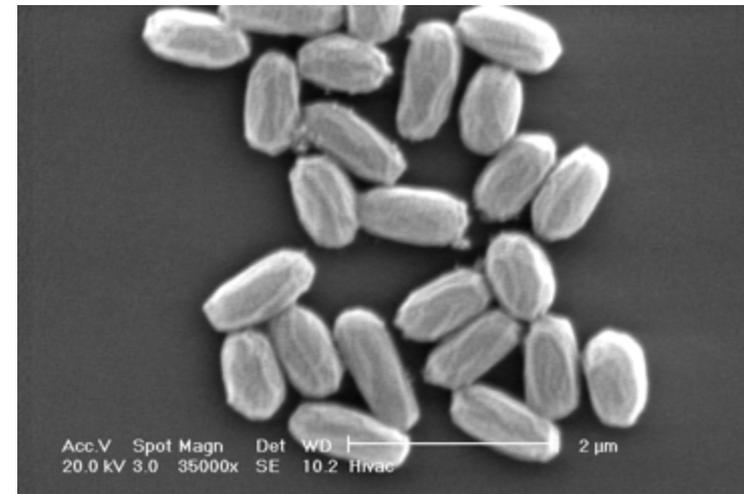
- Bacterial endospores, or “spores”, vary in size but are generally approximately 1 $\mu$ m in size and have been universally used as biological indicators in order to evaluate microbial reduction modalities and sterilization processes.
- Several properties of the bacterial spore make it ideal for examining the efficacy of sterilization procedures.
  - They are typically formed as a response to adverse environmental perturbations
  - They are capable of maintaining desiccation resistance and metabolic stability for months to years.
  - They can potentially transition to a vegetative state under the appropriate growth conditions, and subsequently provide a detectable metric in the form of growth colonies which are easily observed by the unaided eye
- Although spores can survive the chemical and physical challenges which are beyond the capabilities of most other cells, under extreme conditions spores can be killed or otherwise inactivated

# Potential candidates for BI consideration

## Bacterial endospores cont.



- Bacterial spores have been successfully used to evaluate the following processes; dry heat, wet heat, ionizing irradiation (gamma, accelerated electrons, protons, neutrons) non-ionizing radiation (photons, UV wavelength), harsh chemical sterilants, milder chemical disinfectants, freeze-thaw cycles, application of pressure, and exposure to the space environment.
- Spores routinely used as approved BI's for sterilization processes in government, private, and academic settings.
- Use of bacterial spores as BI's for sterilization processes at JPL include:
  - *Bacillus atrophaeus* ATCC 2973 – Dry heat BI
  - *Bacillus* sp. ATCC 29669 – Dry heat BI
  - *Bacillus pumilus* – Ionizing radiation BI
  - *Bacillus sterothermophilus* – Vapor hydrogen peroxide (VHP) BI
- **Thus, we propose the use of bacterial spores as a BI's to assess and evaluate MSR BTC sterilization methodologies.**



*Bacillus pumilus* spores

Image: <https://www.hgsc.bcm.edu/bacillus-pumilus-safr-032>

# Potential candidates for BI consideration

## Viruses and Bacteriophages



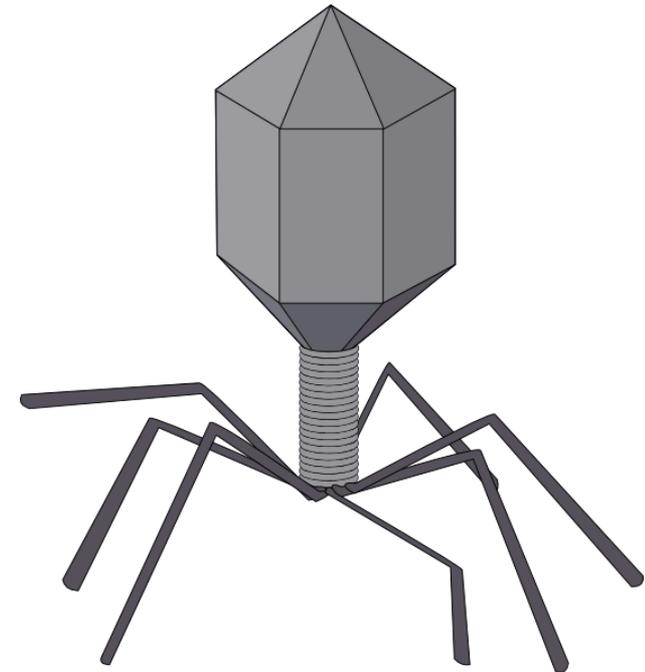
- Viruses are non-living infective agents with either DNA or RNA packaged into a protein coat, with exception of some virus types that also possess an outer lipid membrane.
- Viruses are presumed to infect organisms from all domains of life for replication and synthesis of their metabolic machinery.
- The smallest observed virus, Single-stranded DNA porcine circovirus type 1, is 17 nm.
- Viruses require a host for reproduction, and thus the survival of a “Martian-virus” in return samples requires the presence of a specific “Martian-host” in the sample.
- A rapid evolution specifically targeted toward terrestrial organisms as a host is unlikely unless the virus has a distant ancestral link to Earth.

# Potential candidates for BI consideration

## Viruses and Bacteriophages cont.



- Bacteriophages are viruses that specifically target bacteria
- The sizes of bacteriophages can vary based on the size of their genome, but are generally on the order of 6.5 nm.
- As a candidate in the assessment in our ability to contain a 50nm Mars particle, bacteriophages appear to be a good BI candidate, while also remaining a relevant biogenic organism.
- Bacteriophages are backed by a long history of research and their use in government and industry research (e.g. food, drug, and public health research) is widespread.
- Bacteriophages are relatively easy to grow and maintain with an abundance of options (commercial and private) available for their preparation.
- **Thus, we propose bacteriophages be considered as the size-based BI for MSR BTC containment strategy.**



Generic virus morphology.  
Image: Wiki commons/public domain

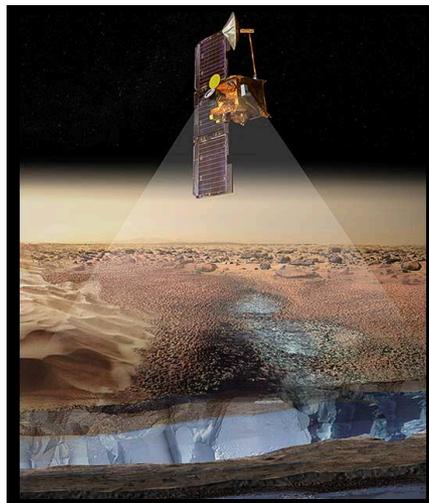
# Other potential candidates for BI consideration



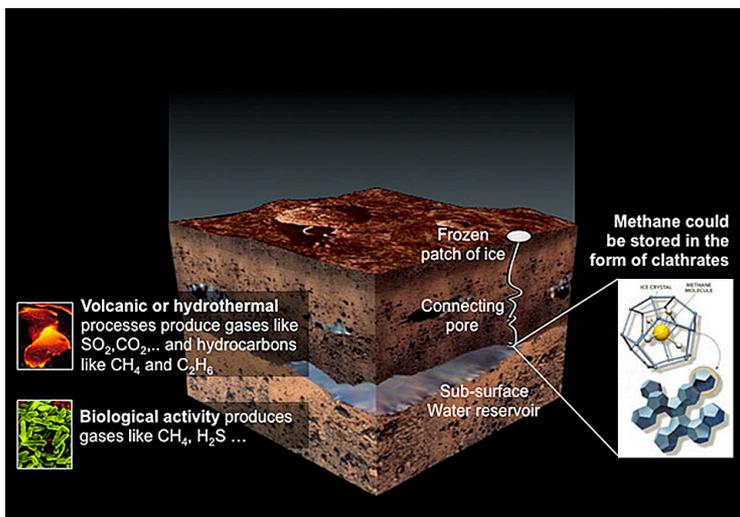
## Possible refugia for Mars life

Mars Sample Return Pre-Formulation

- Mars subsurface
- Endolithic niches
- Ice-water interfaces
- Pockets of liquid brine in the subsurface



Courtesy: NASA



Courtesy: NASA



Pre-Decisional — For Planning and Discussion Purposes Only

# Other potential candidates for BI consideration

## Possible refugia for Mars life



Mars Sample Return Pre-Formulation

- *Evidence for past habitability*
- *Fluvial sediment deposit basin (Fassett and Head, 2005; Ehlmann et al., 2008a; Schon et al., 2012; Goudge et al., 2015)*
- *Sedimentary clays (Ehlmann et al., 2008a, 2009; Goudge et al., 2015).*
- *Mg-carbonates - Olivine-bearing rocks (Ehlmann et al., 2008b, 2009; Goudge et al., 2015)*
- *Fe/Mg smectites*
- *Igneous – basaltic materials*



Mars surface geology  
Courtesy: NASA/JPL/University of Arizona

# Other potential candidates for BI consideration

## Potential pathways to Mars microbial metabolism



- A hypothetical Martian microbe would likely exhibit an **anaerobic redox-driven** metabolism, use a **thermodynamically favorable reduction–oxidation reaction** to generate energy, operate within the **deep- or near-subsurface**. (Nixon et al., 2013)

### Potential metabolic species thought to be present on Mars

electron donors	electron acceptors
Fe <sup>2+</sup> : available in Fe-rich silicates	Fe <sup>3+</sup> : available in numerous alteration minerals
H <sub>2</sub> : available in subsurface?	SO <sub>4</sub> <sup>2-</sup> : available in salts
CO: available in atmosphere	O <sub>2</sub> : partial pressure too low
organics: meteoritic likely to be present at surface	NO <sub>3</sub> <sup>-</sup> : presence or abundance unknown
organics: endogenous available in subsurface	ClO <sub>4</sub> <sup>-</sup> : available but not shown to support growth

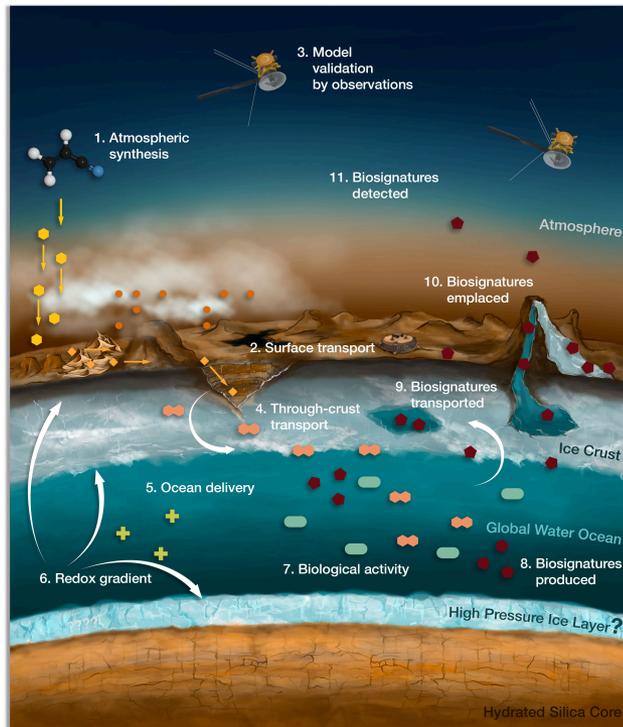
Nixon, S., Cousins, C.R. and Cockell, C., 2013. Plausible microbial metabolisms on Mars. *Astronomy & Geophysics*.

# Other potential candidates for BI consideration



## The redox dilemma

- Mars appears to be rich in electron acceptors for S- and Fe-**reduction** (sulphates, ferric minerals), but lacking in donors (organic carbon, H<sub>2</sub>).
- Electron donors for S- and Fe-**oxidation** (sulphides, ferrous minerals) exist, but electron acceptors such as nitrates are possibly lacking.



- ◆ Surface Sediments
- Atmospheric Fallout
- Potential Life
- ⊕ Inorganic Ions
- ⊕ Delivered Organics
- Volatile Methane
- ◆ Potential Chemical Biosignatures

## Possible candidates

- *Geobacter metallireducens*
- *Thiobacillus ferrooxidans*
- *Acidithiobacillus caldus*
- *Pseudomonas* sp. HerB – Olivine reducing bacteria

Courtesy: NASA

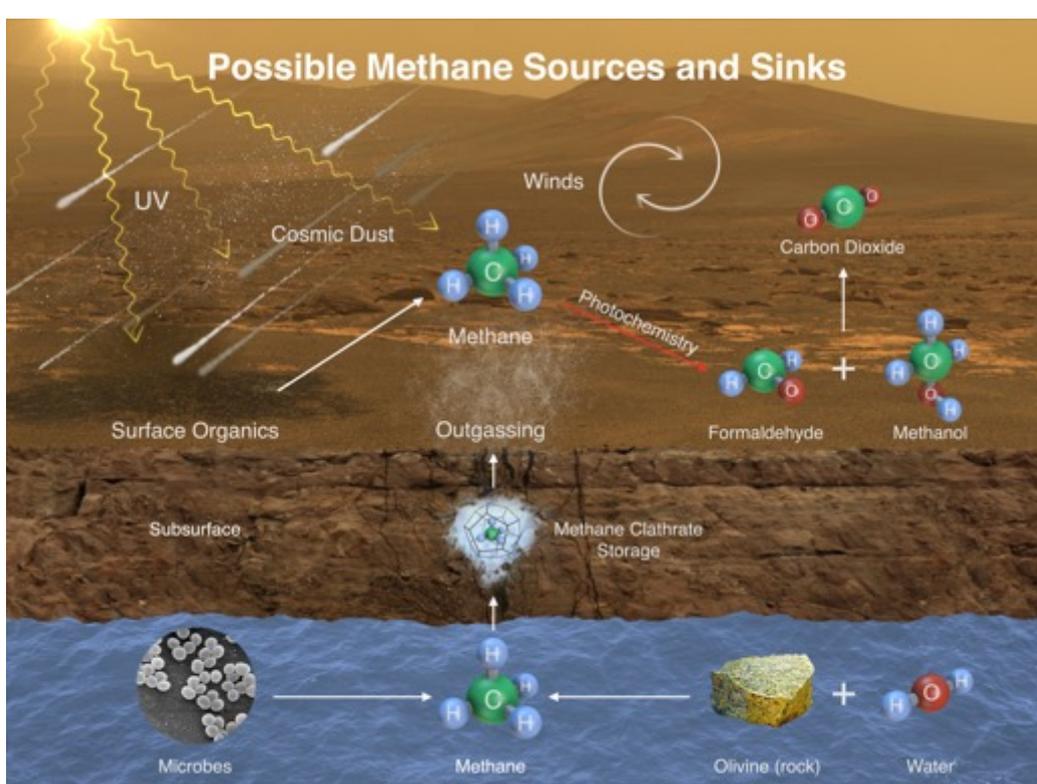
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# Other potential candidates for BI consideration

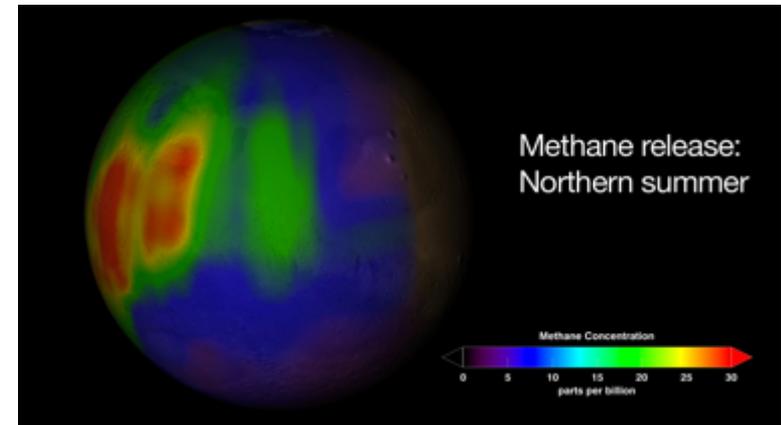


## Methanogens

- Several lines of enquiry over the last few years have indicated the possible presence of localized and seasonally varying methane in the martian atmosphere (Krasnopolsky *et al.* 2004, Formisano *et al.* 2004, Mumma *et al.* 2009).
- Potential Martian sinks of subsurface methane are not fully known.



Courtesy: NASA/JPL



Courtesy: NASA

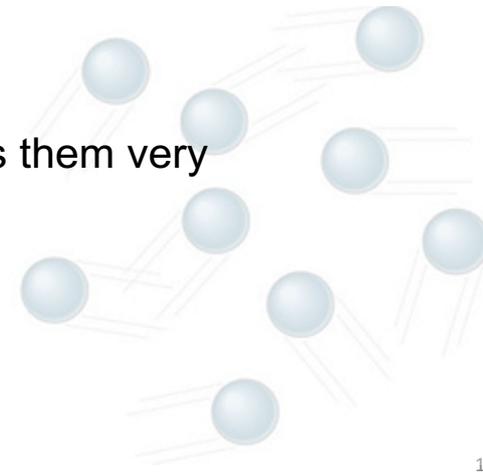
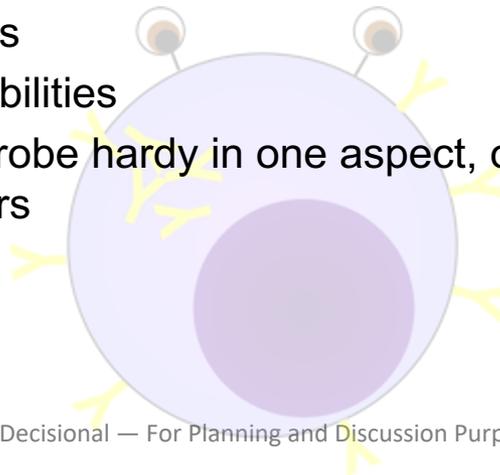
## Possible biological indicators

- *Methanosarcina barkeri*
- *Methanobacterium formicicum*
- *Methanothermobacter wolfeii*

# Summary and additional questions to consider



- We must determine the appropriate range of potential candidate BI's needed to formulate and implement a successful sterilization implementation strategy capable of sterilizing these microorganisms to an acceptable level
- What are the most appropriate BI's for MSR BTC activities?
- Is it appropriate to use our traditional hardy microbe BI's to assess our sterilization strategy, knowing those hardy characteristics (e.g. high heat resistance) may not be representative of those we would anticipate in an extant Martian microbe?
- The BI's selected must be appropriate to the sterilization strategies employed, and conversely, the sterilization strategies employed must also be appropriate and effective to the BI's selected (e.g. using a UV-resistant BI may not be an appropriate verification in a sterilization strategy which does not depend on radiation as its mode of action)
- Some microbial mechanisms to consider:
  - Ability to sporulate
  - Resistance mechanisms
  - Environmental susceptibilities
    - What makes a microbe hardy in one aspect, often makes them very susceptible in others
  - Growth strategies



- We propose that the BI's selected be identified as the most resistant organism/s known relative to the sterilization modality under examination.
  - By identifying the processes that are more than sufficient to kill the most resistant Earth organisms, we maintain that the hypothetical Mars organism would also effectively be destroyed thus ensuring the chain of contact between Mars OS and Earth would be broken.
- We propose BTC technologies utilize bacterial **endospores**, or “spores”, as a BI's to sterilization methodologies and **bacteriophages** as a metric to verify size containment.
  - Bacterial spores have been universally accepted as appropriate BI's to evaluate sterilization modalities and processes.
  - Spores have been successfully used to evaluate the following processes; dry heat, wet heat, ionizing irradiation (gamma, accelerated electrons, protons, neutrons) non-ionizing radiation (photons, UV wavelength), harsh chemical sterilants, milder chemical disinfectants, freeze-thaw cycles, application of pressure, and exposure to the space environment
  - In lieu of bacteriophages, non-viable particles at or below 50nm can also be used if an appropriate detection capability is identified.
- To ensure we remain within effective limits of the each reduction modality, we propose the minimum value of a sterilization parameter (i.e. exposure time, temperature, etc.) that results in complete sterilization be identified, then adding margin (TBD) to that parameter to ensure adequate margin is maintained.

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