480

Utilization of biomarkers to define microbial communities in biofilms and as contaminants of spacecraft searching for extraterrestrial life

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Abstract:

Methods to assess the biological burden carried by spacecraft prior to leaving the Earth are being developed. The demands of the cleaning process for future space missions intended to search for life are far more stringent than the sterilization of viable cells and spores, the standard for the Mars Viking Explorer and other space missions. The experiments to be conducted will target detection of molecules indicative of cellular life, thus any traces of such contaminants carried from earth may compromise the interpretation of the data recovered. Signature lipid and nucleic acid biomarkers to address spacecraft contamination require development of novel molecular methods with levels of sensitivity and molecular specificity considerably beyond those heretofore required for terrestrial samples. Improvements and adaptations of signature lipid and polymerase chain reaction-based analyses are being tested and evaluated with samples from the ultra-clean environment of the Jet Propulsion Laboratory clean rooms where new Mars Explorers are being assembled. This study demands innovation in laboratory practice, including reagent preparation, sample recovery and detection of biomarkers, and in particular new methods for cleaning without leaving life-detection mission compromising residues on the spacecraft.

Aims of the Project:

The aim of this project is to develop and validate methods for detecting both viable microorganisms and non-living cellular components and cell fragments that exist as biofilms and aggregates on the surface of spacecraft and could compromise the detection of extraterrestrial life.

Introduction

Microbes have a long history of being ignored. This is mainly because they are largely invisible and their activities often difficult to observe directly. In addition, the classical methods of isolation and culture of microbes, which were so strikingly successful in the early conquest of infectious diseases taught in most microbiology courses, are not providing answers when applied to bacteria found elsewhere, including environmental surfaces. This is because the great majority of organisms in the environment cannot currently be isolated and grown as monocultures. In general, less than 1% of the organisms

that can be detected in stained microscopic preparations are amenable to cultivation and isolation. Even the use of microscopy has severe limitations. The estimate of 1% recovery given above may be conservative, as in many environments detecting stained microbes attached to or hidden within particulate material may be prohibitively difficult. Methods and agents designed to facilitate the release of attached microbes are often selective and do not release bound microbes quantitatively, so attempts to recover all the biofilm microbiota, including the hidden organisms, are generally compromised. A further weakness of microscopy is that the morphology of the microbes is quite limited without the obvious appendages of the microeukaryotes and morphology does not often reflect the function or activity. Consequently, very little insight into the community structure or nutritional status is possible. Clearly other methods not requiring quantitative growth or recovery of intact organisms are necessary to define the total community composition.

Herein we will focus on two complimentary methods for detecting viable cells and cellular components (biomarkers). Biomarkers to be used in the estimation of biomass should have universality. Equally important, biomarkers should not persist outside the cells if they are to indicate cellular viability. If found in specific subsets of the community, other biomarkers could be utilized to estimate the community composition. Specific biomarkers undergo structural change in particular groups of organisms and this can be correlated to changes in growth status or other physiological processes (1). Biomarkers should be recoverable quantitatively in both reasonable time and at a sensitivity that their detection can indicate small number of microbes. For efficiency the biomarker analysis should allow detection of multiple properties in a single analysis. Unfortunately, no single biomarker class fulfills all these criteria. These demands are compounded in detecting extraterrestrial life, when we are likely to be looking for biomarkers that are actually molecular fossils, indicating that life once existed. We have found that combined analysis of membrane lipids and DNA provides a powerful suite of tools. Together, they have many of the properties of an ideal analytical system to characterize microbial biofilms and aggregates. Methods of sufficient specificity and sensitivity can be generated to be useful in monitoring the contamination of spacecraft whose mission is the detection of extraterrestrial life.

Detection of lipid biomarkers

Over the past 15 years, the laboratories of D. C. White and colleagues in the Center for Environmental Biotechnology have developed signature lipid biomarker (SLB) analyses and have introduced environmental nucleic acid analyses for microbial communities in various environmental matrices. With improvements, these analyses are now being applied to spacecraft contamination. Analysis of the cellular lipids provides a satisfactory way to gain insight into three critical attributes of microbial communities, biomass, composition and physiological status. Lipids are cellular components recoverable by extraction in organic solvents, the extraction providing both purification and concentration. These steps in the SLB analyses are particularly important in achieving sufficient sensitivity to be useful when applied to monitoring spacecraft contamination. On Earth, lipids are an essential component of the membrane of all known cells and play a role as storage materials. If putative Martian life originated in a similar process to that on Earth, it has a membrane to separate the inside of the cells from the outside environment. Consequently the detection of cellular lipids as contaminants is especially important. The lipid biomarker (SLB) analysis will provide quantitative insight into important attributes of microbial communities

SLB is based on the liquid extraction and separation of microbial lipids from biofilms, fractionation of the lipids followed by quantitative analysis using gas chromatography/mass spectrometry (GC/MS) or high performance liquid chromatography/electrospray ionization/mass spectrometry (HPLC/ESI/MS). Several unique classes of lipids, including steroids, diglycerides (DG), triglycerides (TG), respiratory quinones (RQ), poly β-hydroxyalkanoates (PHA), phospholipid lipid fatty acids (PLFA) as well as intact phospholipids, lipo-amino acids, plasmalogens, acyl ethers, and sphingolipids, can be used as SLB to characterize microorganisms or communities of microorganisms (2).

One of the most important SLB classes, phospholipids are essential membrane components of living cells. Because different groups of microorganisms synthesize a variety of PLFA through various biochemical pathways, the PLFA are effective taxonomic markers. Cluster analysis of PLFA has been shown to parallel the phylogenetic relationships between organisms, similar to the higher-level classification revealed by the

more specific phylogenetic analysis based on the sequence homology of 16S ribosomal RNA (3, 4). Knowledge of specific lipid biosynthetic pathways can provide insight into the nutritional status of the microbial community, as certain fatty acids, such as *trans* and cyclopropyl PLFA, provide indications of environmental stress. The analysis was developed for quantitatively assessing microbial communities (bacteria, fungi, protozoa, and metazoa) in slimes, drilling muds, soils, filter retentates, bioreactors, deep subsurface sediments, and also for the detection of specific bacteria in subsurface sediments (5-14). The study of low biomass deep subsurface sediments has provided a strong stimulus for increased sensitivity of detection (15).

Viable Biomass: The determination of the total phospholipid ester-linked fatty acids (PLFA) or the total phospholipid provides a quantitative measure of the viable or potentially viable biomass. Viable microbes have an intact membrane containing phospholipids which are hydrolyzed by cellular enzymes within minutes to hours following cell death with the subsequent release of the phosphate group (16). The lipid remaining is diglyceride (DG). The resulting diglyceride contains the same signature fatty acids as the original phospholipid, at least for some period of time. Consequently, a comparison of the ratio of phospholipid fatty acid profiles to diglyceride fatty acid profiles provides a measure of the ratio of viable to non-viable microbial abundance, and composition of that biomass. A study of subsurface sediment showed that viable biomass as determined by PLFA was equivalent (but with a much smaller standard deviation) to that estimated by intercellular ATP, cell wall muramic acid, and very carefully conducted acridine orange direct counts (AODC) (17). Problems in the estimating the viable cell mass in terms of numbers of microbes from the polar lipid content has been discussed (18).

Community Composition: The presence of some specific groups of microorganisms can be inferred by the detection of unique lipids or combinations of lipids that originate from specific biosynthetic pathways (2). Consequently, the analysis of SLB can provide a quantitative definition of the microbial community composition. For example, specific PLFA are prominent in *Desulfovibrio* sulfate-reducing bacteria, whereas those present in *Desulfobacter* types of sulfate-reducing bacteria are distinctly different (19, 20). The analysis of other lipids such as the sterols (microeukaryotes, 21), glycolipids (phototrophs, Gram-positive bacteria), hydroxy fatty acids (Gram-negative bacteria, 22, 23),

sphingolipids (Sphingomonads, 24), and alkyl ether polar lipids (*Archaea,* 25) can provide a more detailed community composition analysis. When SLB is paired with the analysis of DNA recovered from the same biofilms, the composition of the community can, within the limits of phylogenetic resolution provided by small subunit RNA sequences, be made as specific and extensive as time and resources allow.

Nutritional/Physiological Status: Bacterial poly β-hydroxyalkanoic acid (PHA) (26, 27) and triglyceride in the microeukaryotes (28) are endogenous storage lipids formed when cells have sufficient carbon and energy resources to allow storage. When compared to the PLFA, the relative amounts of these compounds provide a measure of nutritional status. Many bacteria form PHA under conditions of unbalanced growth when a carbon source and terminal electron acceptor (s) is present but cell division is limited by the lack of some essential nutrient (29). Specific patterns of PLFA can indicate physiological stress. Exposure to toxic environments can lead to minicell formation and a relative increase in specific PLFA (30). Increased conversion from *cis* to *trans* PLFA occurs in Gram-negative bacteria with increasingly "stressed" conditions, *e.g.* starvation, desiccation or exposure to solvents (31, 32). Exposure to oxidizing biocides such as chlorine induces the formation of oxirane (epoxy) derivatives in the unsaturated PLFA (33). Oxirane fatty acid formation in the cellular phospholipids correlates with inability to grow on various rescue media (34). Prolonged exposure to conditions inducing stationary growth phase induces the formation of cyclopropane PLFA (30, 35). Respiratory quinone composition can be utilized to indicate the degree of microbial aerobic activity (36, 37). Environments with high potential terminal electron acceptors (oxygen, nitrate) induce formation of benzoquinones in Gram-negative bacteria, in contrast to microbes respiring on organic substrates that synthesize either naphthoquinones or no quinones at all. Some specific but useful insights come from analysis of organisms like the *Pseudomonas* species which form acyl-ornithine lipids when growing with limited bioavailable phosphate (38) and some Gram-positive bacteria form increased levels of acylamino acid phosphatidylglycerols when grown at sub-optimal acid pH levels (39).

Detection of DNA

PCR amplification of rDNA: Ultrasensitive detection of specific microbes can utilize the enormous prowess of PCR enzymatic amplification of DNA. Protocols for recovering DNA from surfaces have been established. When detecting unknown microbes as contaminants, the use of a "nested" series of primers for PCR offers powerful insight because of its exquisite specificity for target molecules. Currently, these code for the bacterial small and large subunit rRNA and microeukaryote small and large subunit rRNA and the more variable linker regions, and permit organism detection at the Kingdom, Family, Genus or Species levels (40). The primer series can be cast widely and a comprehensive analysis can be rendered as specific as necessary for the contaminant detection. The greatest problem in applying these techniques derives from the same factor as its primary advantage, sensitivity. Figure 1 shows amplification products generated from cloned rDNA standards, and demonstrates that, under ideal conditions, 35 cycles of amplification can generate a visible product from <5 template copies. Nested amplification may employ as many as 70 cycles. Clearly, then, the presence of a single rDNA molecule contaminating the laboratory reagents can invalidate a PCR assay. Accepting such a criterion as part of a routine laboratory protocol is, obviously, extremely demanding, and may exceed practical limits (41). By employing procedures more elegant than simple size fractionation, we may be able to obviate this difficulty. Our approach to achieving this is a fortuitous advantage of our method of choice in analysis amplified community rDNA, denaturing gradient gel electrophoresis (DGGE) or thermal gradient gel electrophoresis (TGGE). These methods allow DNA fragments of the same length to be separated according to their melting properties, which are inherent properties of their primary sequences. Double stranded DNA is separated in a linearly increasing denaturing gradient of urea and formaldehyde at elevated temperatures during DGGE (42). In TGGE the double stranded DNA is separated using a linearly increasing temperature gradient in a uniform concentration of urea and formamide (43). With either technique, mixed amplified PCR products form a banding pattern. The banding patterns generated from reagent-controls can be compared to the banding patterns produced from test samples, and the common bands ignored (Figure 2). Utilizing rRNA genes DGGE/TGGE analysis of PCR products has provided structural diversity of communities that has proved reproducible (43-45). The major advantage of PCR-DGGE/TGGE is that the *bona fide* bands generated

from test samples can be isolated and sequenced and the sequences compared to sequence databases to establish phylogenetic relationships. If specific groups of organisms are found to provide the most common contaminants, specific primers may be designed to eliminate laboratory contaminants such as is done when detecting minor components of complex microbial communities such as *Nitrosomonas* and *Nitrospira* communities in soil (45). Once the typical contaminants of a spacecraft assembly clean room are known, it may be possible to employ primers to focus on detection of specific non-ribosomal targets. This may allow the development of novel primers with enhanced sensitivity as compared to 16S rDNA primer pairs, thereby allowing a reduction in PCR cycle-number, further reducing the danger of laboratory contamination.

One of the major failures of these powerful molecular techniques for analysis of environmental samples is the lack of a quantitative aspect to interpretation of the PCR amplified material. PCR is commonly rendered quantitative by the addition of a competitor molecule at known copy number into the reaction tubes. The amplification products are then separated in some way, usually by size. We have adapted this technique (46) to count the total number of rDNA molecules present in environmental samples (Figure 3). We have also extended this principle to PCR-DGGE analysis, by the incorporation of a competitive standard which denatures at a unique position in the denaturing gradient (Figure 4; 46). Serious problems still remain in PCR methodology. The accuracy of these methods is largely dependent on DNA extraction efficiency, which partially depends on quantitative cell lysis. It has been repeatedly demonstrated that components of soil microbiota are differentially lysed by chemical and physical methods (47). Gram-positive bacteria such as actinomycetes, various cocci, spores, and yeasts may be particularly difficult to lyse and subsequently extract (48). These are the same microbes most frequently recovered by viable plate count assay in clean rooms typical for spacecraft assembly systems (49). In addition, fractionation of the microbial biomass prior to DNA extraction introduces a bias towards those organisms which are easily dislodged from the surface resulting in non-representative sampling. The polyphenols, tannins, iron chelates, and clays that make the recovery, amplification, and detection of DNA (by DNA:DNA hybridization) difficult from soils are, however, much less of a problem with spacecraft surfaces. For example, although PCR is theoretically capable of detecting one target molecule in a sample, impurities

present with DNA extracted from soil can lower the sensitivity of PCR to 10^4-10^8 cells per gram of soil (50). Furthermore, during enzymatic processes such as PCR other restrictions such as primer choice (50), PCR conditions (51-54), PCR drift and selection (55) and cloning (51) may be introduced, again biasing results. PCR selection occurs when a reaction favors certain members of a gene family (i.e. separate reactions produce results skewed towards the same genes) while PCR drift is a bias that occurs as a result of random events in the early cycles of the reaction. PCR drift can be countered by carrying out several reactions and pooling the products to reduce any skewing of the results caused by the independent random PCR events (55). In addition, PCR selection can be countered by carrying out the reaction using the smallest possible number of amplification cycles (55). Recent evidence has shown that genome size and the number of rRNA genes (rDNA) present per cell will impact on the species represented in clonal libraries of environmental samples (56). Another major disadvantage of PCR when applied to community structure analysis and organism identification is the formation of chimeric sequences, that is hybrid molecules made up of two different 16S rDNA types (57). The formation of chimeras introduces risk when using PCR to examine microbial community structures. As such, critical analysis of sequences using a computer program such as Check Chimera (58) is vital to any interpretation of the data. Heteroduplexes are formed when two single stranded molecules from different organisms anneal. Cloning of such a construct in *E. coli*, a common procedure, induces the *E. coli* mismatch repair mechanism to synthesize a perfect duplex, with a novel sequence not identical to that of either source organism. These problems increase with the number of rounds of amplification, again emphasizing the necessity for PCR optimization. In our studies, we avoid procedures involving molecular cloning, preferring direct sequencing of bands excised from DGGE gels.

The powerful quantitative assessment SLB method developed over the past 20 years by this laboratory defines viable biomass, community structure and nutritional/physiological status of environmental microbial communities. This has now been expanded to include defining the proportions of specific genes or microbial species based on the extracted DNA. The combined DNA/lipid analysis overcomes some deficiencies in microbial ecology studies involving only nucleic acid analysis (59).

Analysis of air samples: Recently the ability to recover microbes from reproducibly generated aerosols has enabled us to show that the SLB analysis system is orders of magnitude more effective than the classical plate count or liquid trap with viable count methods (60). Biomass collection on glass fiber filters followed by SLB analysis has been utilized for monitoring industrial indoor air biocontaminated systems (61). However, large volumes of air are necessary to generate enough biomass when utilizing SLB detection of microbial aerosols. Introduction of the PCR-DGGE analysis has allowed lower volume sample collection utilizing all glass impingers and liquid as the capture medium. Figure 2 illustrates the PCR-DGGE profiles of rDNA from clean-room air and outdoor air obtained using this system. In this example, no detectable bacteria were recovered from the clean-room air, whereas a single major species, was detected in the outdoor air. The bands recovered from the reagent blank, filter blank, and clean-room air were identical, and the same bands were also visible in the sample recovered from outdoor air, as well as the single bona fide sample-generated amplification product. This emphasizes the necessity of carefully analyzing the amplification products, as with large numbers of cycles the impurities in the reagents become an ever increasing problem.

Increasing the rate and recovery of SLB/DNA extraction: Utilization of an automated, commercially available accelerated solvent extraction system (ASE 200, Dionex corp.), to extract glass fiber filters exposed to indoor air has been compared with the standard one-phase chloroform/methanol/aqueous buffer extraction (62). The one-phase modified Bligh and Dyer solvent extraction is a commonly used method for obtaining phospholipid fatty acid biomarkers used in such community analysis. This method, however, is labor intensive and slow, often taking up to 24 hours for the initial extraction. Using a pressurized hot solvent extractor, we have been able to extract lipid biomarkers from selected vegetative and/or sporulated biomass (*Escherichia coli*, *Staphylococcus aureus*, *Mycobacterium fortuitum*, *Bacillus subtilis*, *Saccharomyces cerevisiae* and *Aspergillus niger*) as well as from environmental samples collected from water, soil and air. Dependent on sample type, the automated extraction procedure took approximately 35-45 minutes *per* sample. Compared to the modified Bligh and Dyer extraction, phospholipid fatty acid lipid yields obtained using the pressurized hot solvent extraction were not significantly different for the vegetative biomass or water and soil samples, but were

significantly higher for the spores (200%) and the air (300%) samples. The advantage of using accelerated hot solvent extraction is that by increasing the speed and decreasing the labor involved, pressurized hot solvent extraction should enable the rapid and improved extraction of lipids from large numbers of environmental samples providing data essential for total microbial community analysis. The high temperature/pressure extraction system can be automated with several commercial systems.

Bacterial Spore detection: 2,6-Dipicolinic Acid (DPA) is a unique biomarker for bacterial spores. It has been shown that numerous bacteria contain genes for DPA synthesis but are unable to form spores (Carol Smith, unpublished). Consequently, detection of DPA itself is by far the best method to detect spores. *In situ* derivatization and extraction using supercritical fluid extraction (SFE) has been shown to allow recovery of DPA from bacterial spores at heretofore unachievable efficiencies (63). With subsequent analysis by GC/MS it proved possible to detect 2,6 dimethyl dipicolinate with selective ion monitoring of three major ions of the following m/z values and relative abundance [m/z (relative abundance)]: 165(65), 137(100), 105(16) at levels equivalent to less than a 100 bacterial spores. Fragment ions monitored correspond to a fragment resulting from the loss of a methoxy functionality from the protonated molecular ion (m/z = 165), the loss of methyl formate from the protonated molecular ion (m/z = 137), and, finally, the loss of methanol as a neutral loss from the base fragment ion at m/z = 137 (m/z = 105). The detection and control of bacterial spores is critical to Planetary Protection as the current legal standard based on the Viking protocol for spacecraft sterility allows up to 3000 viable spores/m^2 spacecraft surface or 30,000 viable spores/spacecraft. Clearly this standard is far too large for life detection science missions.

Recent improvements in the detection technology:

Increasing the sensitivity of phospholipid detection:

Phospholipids as biomarkers provide some advantages for sensitive detection. They are present in relatively high concentration, are easily extracted from any number of complex matrices, and are extremely diverse. The diversity of phospholipids is due to the large number of possible combinations concerning the identity and position of fatty acyl substituents as well as the nature of the polar head group. Current methodologies for the

analysis of phospholipids, which utilizes gas chromatography/mass spectrometric instrumentation, rely upon mild alkaline methanolysis of formerly acylated fatty acid constituents yielding fatty acid methyl esters (FAME's) that are more amenable to gas chromatographic analysis. This assay of the polar lipid fatty acids (PLFA) provides an immense amount of information regarding the quantity of viable microbial biomass present, the identity and diversity of the community composition, as well as monitoring the nutritional or physiological status of the microbial community. However, upon hydrolysis, information regarding the identity of the endogenous phospholipid, such as the nature of the polar head group and regiospecific information regarding the identities of the two fatty acyl constituents, is lost. With the loss in structural information inherent in this technique is a concurrent loss in potential for chemotaxonomic differentiation. Therefore, a complementary method for the qualitative and quantitative analysis of intact, endogenous phospholipids using HPLC/ESI/MS has been developed. Not only is the specificity increased using HPLC/ESI/MS but also the absolute sensitivity of the assay is increased from approximately 1 pmol of PLFA for (GC/MS) to approximately 1 fmol of phospholipid for the microbore HPLC/ESI/MS analytical system. .

HPLC/ESI/MS offers the advantages of little sample preparation, speed of analysis, extremely high sensitivity, as well as providing sufficient information for structural verification. The sensitivity of the assay has been established in the high attomoles of material providing "working" chromatograms from low femtomoles of analyte resulting in very little sample consumption. The remaining sample may then be reacted with a suitable derivatization reagent for GC/MS analysis. Separation of all classes of phospholipids is accomplished online within 40 minutes utilizing a mobile phase consisting of methanol/water (92.5:7.5) and a microbore reversed-phase C18 column. Structural information varies with mode of ionization. In the generation of positive ions, 0.5 mL/L of formic acid is added whereas in negative ionization mode, 0.5 mL/L of aqueous ammonia is added. In positive ion mode, protonated molecular ions are observed for all classes of phospholipids. At elevated cone voltages (125V), loss of the polar head group as a neutral species with charge retention on the remaining fragment results in information regarding the nature of the polar head group by mass difference (along with retention time). The notable exception to this rule is phosphatidylcholine (PC) whose polar head group

fragments with charge retention producing a base peak at a m/z value of 184. Additionally, constant neutral mass loss scans in the positive mode of ionization results in the identification of those molecular ion currents belonging to a particular class of phospholipid without the need for prior chromatographic separation. We have also demonstrated the sensitivity of this method to be ~300 attomoles of phospholipid with a borrowed but un-optimized Quattro II triple quadrupole mass spectrometer. Negative ion electrospray ionization of phospholipids is much more structurally significant than is the previously described positive mode of ionization. In the negative mode of ionization, all classes of phospholipids produce a deprotonated molecular ion $(M-H)^-$, again with the exception of phosphatidylcholine, which produces a demethylated ion $(M-CH_4)^-$. At elevated cone voltages, fragment ions corresponding to the two fatty acyl constituents are prominent as are low mass ions diagnostic of the polar head group. Fragmentation processes are also regiospecific, in that fragmentation resulting in fatty acyl ions from the sn-2 position is favored over fragmentation at the sn-1. This is reflected in the ion abundance values of these two species. Stereospecific information relies upon tandem mass spectrometric instrumentation, which unfortunately is currently unavailable at the CEB. However, our current system, which relies upon a single quadrupole mass analyzer, does provide additional information that was previously unattainable through GC/MS PLFA methodologies. Information regarding the nature and quantities of phospholipid classes as well as information regarding the nature and quantity of fatty acid constituents specific to a particular class of phospholipid demonstrates the increased potential of this technique at far better sensitivities (100-1000 fold greater sensitivity) than was previously obtainable. The addition of a triple quadrupole mass analyzer would result in a more sensitive and specific SLB methodology capable of assignment of absolute structural characteristics of intact, indigenous phospholipids and, thus, obtaining far greater chemotaxonomic information. Additionally, the mass filtering capabilities should result in lower limits of detection due to the reduction of chemical noise. The ability to fragment specific parent ions in the collision cell (rf only quadrupole) rather than in-source by elevation of the cone voltage applied is much less specific resulting in fragment ions attributable to any co-eluting precursor ion. Monitoring of total ion currents in the constant neutral mass loss scans would result in quantitative and qualitative information regarding the class of phospholipids present in a

sample without the need for HPLC purification. Finally, daughter ion scans of those deprotonated molecular ions identified as belonging to each class of phospholipid by constant neutral mass loss scans would result in the regiospecific characterization of each molecular species present, again, without the need for prior HPLC purification at attomole sensitivity. Selected reaction monitoring (SRM) capabilities would result in much more specific or selective assays for the respiratory quinones, dipicolinic acid, and oxirane PLFA with enhanced sensitivity. The sensitivity for the analysis of bacterial phospholipids between GC/MS, HPLC/ESI/MS, and HPLC/EIS/MS/MS is compared in Table 1. Several points concerning this table should be addressed. First, those values presented do not necessarily reflect the limit of detection (LOD) by each technique. Rather the lowest quantity detected is reported with the signal to noise to value. The value reported for GC/MS analysis does correspond to the LOD. Values reported by LC/MS techniques roughly correspond to the limit of quantitation (LOQ), a more useful term, by each technique. Finally, it should be evident that the sensitivity gained by utilization of LC/MS methodologies approaches a 1000-fold and with LC/MS/MS 10000 fold.

Developing in situ on-line detection

The efficacy of the combined complementary SLB and PCR-DGGE of rDNA for detecting contamination on spacecraft have been discussed and remarkable specificity and sensitivities achieved. Currently we are actively pursuing the development of on-line monitoring system based on detection of ultraviolet fluorescence (Emission at 330-370-nm), of tryptophane in proteins (Excitation at 285-290 nm) and DNA (Excitation at 250-260 nm) which could provide a detailed history of contamination. This would supplement the sensitive destructive ultrasensitive methods described above and provide rapid feed-back during spacecraft assembly.

Cleaning

Once the contamination with biomarkers that could compromise the identification of extraterrestrial life are detected, the contaminants must be removed or rendered non-compromising. In the Viking missions the search was for life and metabolic activity so the requirements were that contaminants be sterilized to not grow or be metabolically active. With the current biomarker detection requirements it is still important that the contaminants

not reproduce or generate metabolic products. Sterility is important. However the non-viable, non-metabolically active biomarker residues of contamination must now be considered just as capable of compromising the science goals of the mission as a viable spore would be to Viking. Cleaning surfaces free of contamination and maintaining cleaned surfaces now becomes the critical consideration.

Two cleaning technologies are currently under investigation.

Recovery from the surface: The current standard NASA cleaning procedure is to scrub the surface in two directions with a swab and solvent of 70% aqueous ethanol. In Figure 5 the inadequacy of this cleaning is illustrated. Wiping an air dried suspension of *Mycobacterium pflei* engineered to contain green fluorescent protein (GFP) with 70% aqueous ethanol in two orthogonal directions with a Kimwipe, lysed the cells but did not remove the GFP. Similar experiments with other bacteria show the cellular DNA is not removed but mostly smeared over the surface with somewhat less than 10% removed from the surface. All the standard solvent wipe cleaning did was lyse the cells, achieving sterilization, but not removing the biomarkers. When the NASA standard cotton swab was used, the surface after cleaning contained cotton fibers that were readily detected microscopically not present before the cleaning. .

It is so difficult to quantitatively recover contamination from spacecraft surfaces that much of the research has focused on coupons or witness plates that can be recovered and processed. Currently adhesive tapes for contaminant removal are being tested based on the success of 3M tape 5414 in the recovery of fingerprints. The tape itself leaves a residue which may prove tolerable if it is not volatile under launch and landing conditions and the residue chemistry is distinctly different from that of possible biomarkers. Experience in the electronics industry has indicated that the only effective cleaning of silicon wafers involves mechanical cleaning. Brushing with ultrapure water will be tested using brushes that leave no residue that could be confused with possible biomarkers.

In situ destruction: Cleaning methods that involve *in situ* destruction are being examined. Treatment of spacecraft with high pressure hydrogen peroxide looks promising. In this case the sterilizant does not leave a residue other than water and carbon oxides. Plasma treatments and effects of ultraviolet light on surfaces designed to enhance contaminant destruction be are also being tested. In all these treatments the chemistry of

the post-treatment residue must be defined and as far as possible be distinctive from possible extraterrestrial biomarkers. Heat treatments of 113° C for many hours as were utilized in Viking are not compatible with the newer spacecraft materials and instruments.

Spacecraft components that cannot be cleaned effectively are sealed behind biobarriers but these biobarriers must have adequate filters to compensate for the pressure changes endured during launch, transit in space, and landing. Further, the absolutely critical areas where samples are recovered and cachet must be protected from less well cleaned areas of the spacecraft to prevent contamination transfer from one part of the spacecraft to another in 200 mile-an-hour Martian dust storms.

Clearly Planetary Protection is crucial in the answer to the search for the first positive indication of extraterrestrial life on the only planet from which we can expect samples to be returned in our lifetime. We must not have an equivocal answer to the question of whether there was (or is) life on Mars in 2008 because a contaminating terrestrial biofilm was taken to the planet.

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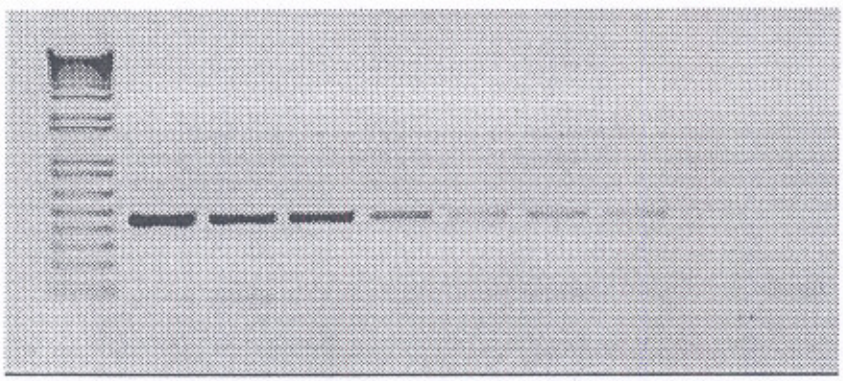
Legend to Figure 1

Detection limits of 35 cycles of PCR amplification with ethidium bromide staining.

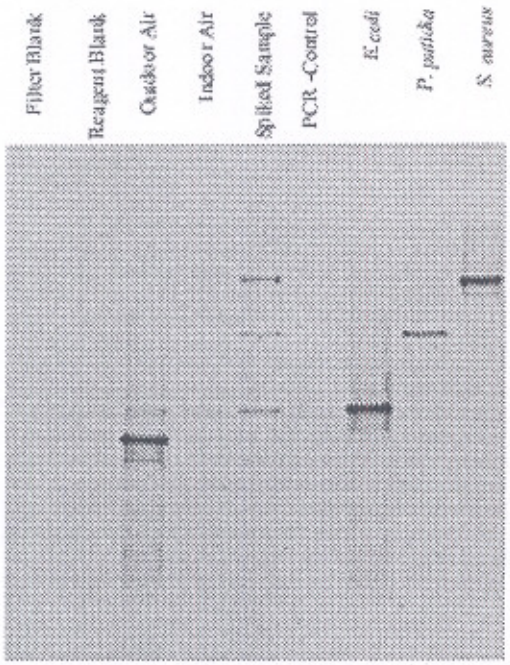
A standard dilution of a cloned, purified and quantified (Hoefer DyNA-Quant 200 Fluorometer and Hoechst H33258 dye binding assay) 16S rDNA fragment was subjected to PCR-amplification to generate a 450 bp fragment. *Via* ethidium bromide staining, a product could be visualized from 1-5 copies of template DNA. From left to right. Molecular weight marker, 104, 103, 500, 250, 100, 50, 10, 5, 0 copies of target DNA. Figures are maximum values (95% confidence).



Legend to Figure 2

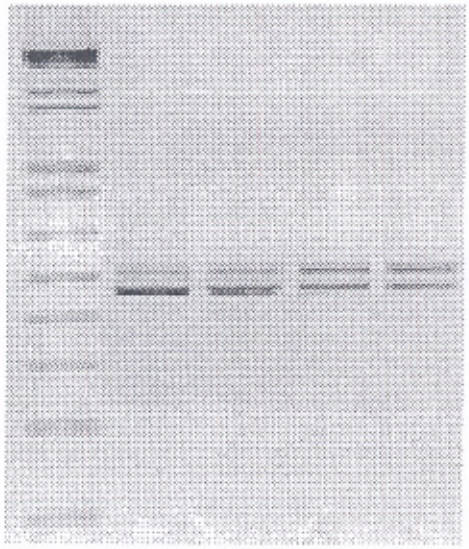
PCR-DGGE analysis of clean-room and outdoor air

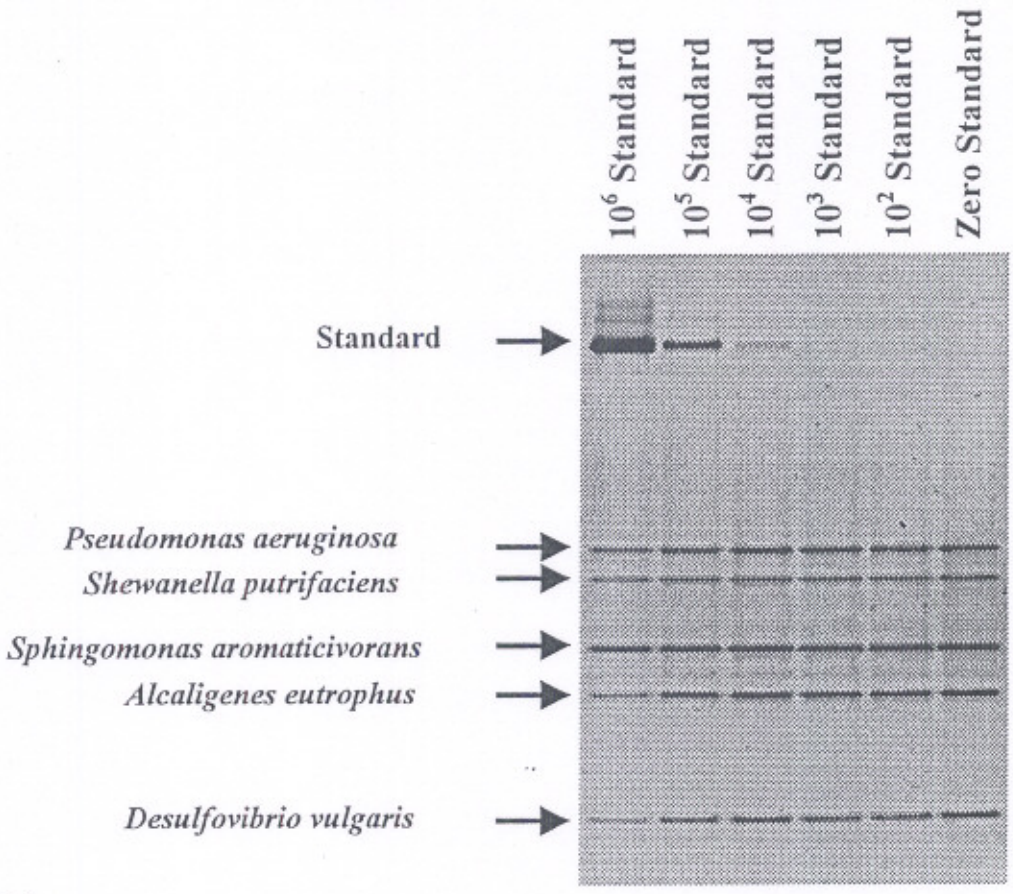
In this experiment, clean-room, outdoor air and indoor air spiked with an aerosol containing *Escherichia coli, Pseudomonas putida* and *Staphlococcus aureus* was collected by all-glass impinger. DNA was extracted from the impinger water by bead-milling. PCR-DGGE was as performed (42). Negative controls recovered a single band, which co-migrated with the *E. coli* amplification product. *E. coli* is a common lab contaminant.



Legend to Figure 3

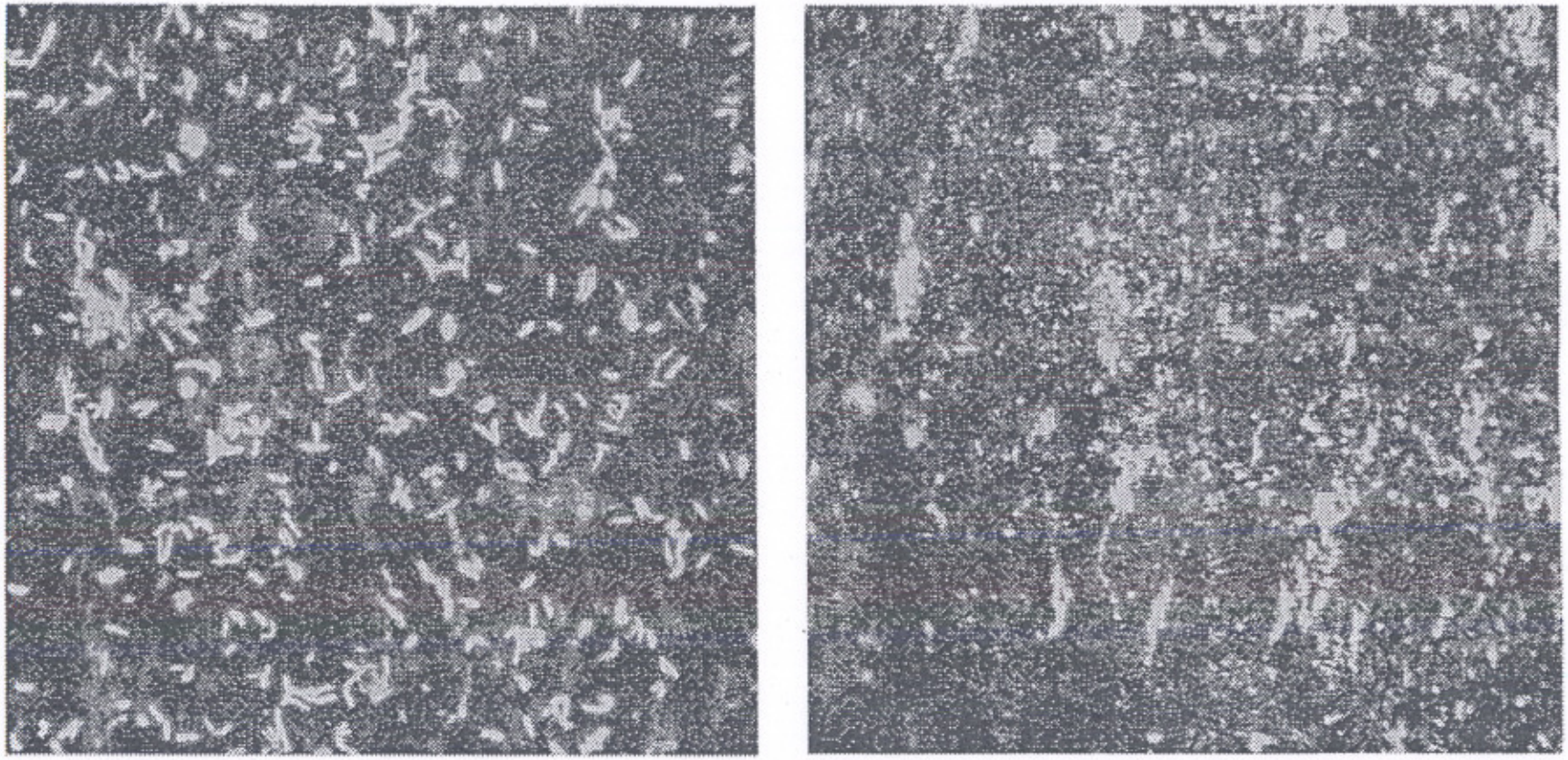
Quantitative PCR of total rDNA. A deletion fragment of a 16S rDNA molecule was constructed to be slightly shorter than any published 16S rDNA fragment spanning the same conserved PCR-priming sites. The standard was quantified and added to a DNA extract containing unknown amounts of bacterial DNA. The ratio between the amount of wild-type rDNA and deletion fragment recovered after PCR is an accurate measure of the starting copy-number in the DNA extract. From left to right. Molecular weight marker. Unknown plus 2000, 1000, 500 and 250 copies of competitor.





Legend to Figure 4

Multiple-competitive quantitative PCR-DGGE.
As in Fig. 3, the starting copy-number of each species detected by PCR-DGGE can be estimated by reference to the internal standard. The example shown was an experiment wherein the amount of test DNA, consisting of genomic DNA extracted from a mixture of 5 bacteria, was held constant, and the number of standard copies



A B

Legend to Figure 5.
Effect of wiping Aluminium 6061 on which *Mycobacterium pflei* containing green fluorescent protein had been air dried. Surface was wiped Kimwipe in two dimensions with a solvent of 70% aqueous alcohol. A. Before wiping. B. After Wiping. Taken with a confocal microscope and detected by fluorescence (excitation ~395nm, emission ~509nm) The horizontal field is ~220 um long.

Table 1: Lowest Quantity Detected of Synthetic Phosphatidylethanolamine (PE) and Fatty Acid Methyl Esters (FAME's) Derived from Phosphatidylethanolamine by Four Distinct Mass Spectral Techniques

Mass Spectral Technique	Scan Mode	Compound Analyzed	Lowest Quantity Detected	Signal to Noise Ratio
GC/MS	Full	FAME	460 fmol	4:1
LC/MS (single quad)	Full	PE	960 amol	10:1
LC/MS (triple quad)	Full	PE	280 amol	10:1
LC/MS (triple quad)	Constant Neutral Loss	PE	210 amol	8:1