Microbial Contamination of Allende and Murchison Carbonaceous Chondrites; Developing a Protocol for Life Detection in Extraterrestrial Materials using Biotechnology.

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Abstract

The arguments used to refute the McKay et al., (1996) hypothesis of possible Martian life in ALH84001 failed to use contamination of the meteorite as a source. This has worrying implications for our ability to detect terrestrial microbiota in meteorites and therefore any potential extraterrestrial biosignatures in both meteorites and possible returned samples. We report on imaging and microbial culturing of both Allende and Murchison carbonaceous chondrites and on the use of molecular biology techniques on a sample of Allende. Contaminating fungi and bacteria were observed (in the case of Murchison) and cultured from both meteorites. DNA was successfully extracted and subsequent PCR showed the presence of both bacterial and fungal DNA although no Archaea were detected. These results show that it is possible to use molecular biological techniques on very small quantities (300 mg) of extraterrestrial material.

Introduction

The argument over life in ALH84001 has had some major implications for the search for life elsewhere in our solar system [1]. The foremost of which is that although this meteorite contains evidence of terrestrial biological contamination the presence of these organisms was not detected by methods used to refute the McKay hypothesis [2,3,4,5]. In general terms it would appear that we have a basic inability to detect small numbers of a microbial species in an organic poor environment. In an attempt to classify further the presence of contaminating organisms on two carbonaceous chondrites (Allende and Murchison) a combination of imaging, microbial culturing and in the case of Allende direct DNA extraction was used.

Our approach has been one that evolved through the process of studying first the Murchison meteorite and then Allende. Initially light microscopy was performed on Murchison, from this, culturing and then scanning electron microscopy (SEM) of the same sample (plus a control sample) was undertaken. Due to the results of the culturing studies, microscopy, culturing and also direct DNA extraction / amplification was undertaken on the Allende samples. This was achieved using the Polymerase Chain Reaction (PCR) with primers for the detection of Eubacteria, fungi and Archaea bacteria. Bacterial 16S rDNA sequence analysis and fungal 18S rDNA sequence analysis, has been successfully applied to pure cultures isolated from Allende and is currently underway on the direct DNA extraction products (these results will therefore not be shown in this paper). The molecular biology approach was undertaken in an attempt to classify all species present in the meteorite and not just those that could be cultured on only a small range of medias. It is hoped that by using this approach we can identify the bacterial species involved in contamination and therefore trace the principle sources of contamination and understand more fully terrestrial microbial metabolism within the meteorites. Furthermore, detection of contaminating terrestrial microbiota is very analogous to the problems of detecting potential

extraterrestrial life within meteorites and Martian return samples. By using the meteorites as a potential model, techniques to search for life elsewhere in the universe can be tried and tested.

Materials and methods.

Meteorites

Two chips of the Murchison meteorite were examined, the main focus of the research was conducted on a chip of Murchison from the Field museum in Western Australia supplied by R. Hoover. This chip did not contain any evidence of fusion crust and apparently originated from a fresh fracture surface in an area beneath the fusion crust (named sample 1, Mu1). The second sample (named Mu2) of Murchison was from an allocation supplied by D.S. McKay and had been previously gold coated for imaging. This sample was only used as a Scanning Electron Microscopy (SEM) control for studies conducted on sample 1, and had been stored in a sealed plastic SEM storage container, curated by D.S. McKay for approximately 15 years.

The Allende sample was also supplied by D.S. McKay and measured approximately 2 cm square and 2 cm deep and has an uncertain curation history. The single sample was aseptically split within a laminar flow cabinet (using sterilized tools and within sterile petridishs) to separate surface material from internal material (samples designated Aint, Aext for internal to the chip and external to the chip respectively). Samples were then stored in sterile petri-dishes.

Imaging

Light microscopy was first undertaken (in laminar flow conditions) on the Murchison sample but not on Allende. Light microscopy imaging of Mu1 was conducted using a Leitz reflecting light

microscope equipped with a photoautomat and 35 mm camera. The sample was removed from the sample container in laminar flow conditions and placed on a sterile petridish using aseptic technique. The exposed surfaces of the microscope (placed within the laminar flow hood) were swabbed with alcohol before imaging to remove any loose dust contaminants. After examination the chip was sealed within the petridish.

SEM imaging was conducted on Mu1 only after completion of the culturing experiments and on isolated chips of both the internal and external surfaces of the Allende chip. The samples were subjected to a standard biological SEM preparation; the sample was fixed in 2.5% (v/v) glutaraldehyde in phosphate buffer (0.01 M) at 4°C for 24 hours. The sample was then dehydrated in a series of 30, 50, 70, 90, and 100% ethanol / water series (15 minutes in each solution), then rapidly frozen in liquid nitrogen, transferred to a lyophilizer and freeze-dried at -70°C under vacuum overnight. The freeze dried sample was then mounted on aluminium supports, sputter coated with Au / Pd for 30 seconds and examined under a field emission gun Scanning Electron Microscope (Philips XL40S, FEG-SEM) at various acceleration voltages (3 - 25 kV). The Mu 2 sample was removed from its storage container and remounted on a SEM stub suitable for the Philips XL40S, and then re-coated with Au / Pd for 15 seconds before SEM imaging.

Culturing experiment

Due to light microscopy examination of the Mu1 sample revealing the presence of a possible fungi (see Figure 1) culturing was attempted. The whole Mu1 sample was pressed (using sterile tweezers) into Sauberaut dextrose agar (Oxoid, UK), with the noted biogenic features entering the medium and incubated aerobically at 28°C for 2 weeks. After this time the resulting organisms on

the plate were streaked onto fresh medium and any bacterial isolates were then further sub-cultured and identified using gram stain, morphology and API microbial (Biomereaux, France) identification tests. The microbial species cultured during this experiment represent only a tiny fraction of the possible microbial content of the samples as only aerobic species with a high nutrient requirement were cultured for. After this time the chip was removed from the old medium and pressed into fresh Sauberaut dextrose agar and further incubated at 28°C, until the medium had dried out (6 weeks). This was done in an attempt to impose harsh environmental stress on any isolates colonizing the surface (i.e. loss of nutrient source and dehydration). This chip was then prepared for SEM investigation as described above.

For Allende a suspension of both the interior and exterior of the sample chip were prepared aseptically by grinding the chips (approx 300mg in each case) and adding 30ml of sterile ultrapure water. To remove bacterial cells from the particulate matter the sample was vortexed for 1 minute then allowed to stand for 1 minute. This was repeated 5 times so that the meteorite suspension was vortexed for a total of 5 minutes. Bacterial cultures were performed on the following media; nutrient agar, artificial soil media containing cyclohexamide (50 mg ml^{-1} final concentration) for suppression of fungal growth, and the minimal R2A media. Potato dextrose agar was also used to isolate any fungal and yeast species. All plates were inoculated with 100 µl of the meteorite suspension and incubated at 35°C for 72 hrs. Following incubation, the isolates obtained were sub-cultured (to obtain pure cultures) on to fresh plates of the same media and also inoculated on to nutrient agar plates. All plates were subsequently incubated at 30°C for 4 days.

SEM investigation was conducted on chips of all three samples (Aint, Aext and Aenr), samples were prepared as described previously.

Direct DNA extraction / amplification of the Allende sample

Overnight cultures were performed by inoculating 1 ml of the meteorite solution into 10 ml of nutrient broth and incubated at 30°C. This was done to ensure high microbial concentrations in an attempt to ensure suitable quantities of DNA were present to enable direct DNA extraction (designated Aenr). After incubation overnight, the resulting microbial suspension was centrifuged at 13,000 rpm for 10 minutes and the resulting cell pellet resuspended in 0.75ml of 0.12 M NaH_2PO_4 containing 1% Sodium Dodecyl Sulphate and 0.5ml phenol / chloroform / isoamyl alcohol (25:24:1 v/v/v) pH 8.0.

The extraction method was performed on samples from the interior and exterior and from the enrichment culture as well as any pure cultures isolated from the culturing experiments. The meteorite suspensions (4 ml) were centrifuged 13,000 rpm for 5 minutes. The resulting cell pellet was re-suspended in 4 ml of sterile ultrapure water and then vortexed for 1 minute. Cell lysis was performed using a ribolyser (Hybaid) for 30 sec (setting 6), according to the manufacturers instructions and then centrifuged at 13,000 rpm for 5 minutes. The aqueous phase was removed and an equal volume of phenol / chloroform / isoamyl alcohol (25:24:1 v/v/v) at pH 8.0 was added. The sample was vortexed for 1 minute and centrifuged at 13,000 rpm for a further 5 minutes. The aqueous phase was retained and the nucleic acids precipitated for 24 hours using an equal volume of 30% polyethylene glycol 6000 and 0.1 volume 5M NaCl. Following precipitation, the nucleic acids were pelleted by centrifuging 13,000 rpm for 5 minutes and washed with 80% ethanol.

Eubacterial PCR amplification of meteorite DNA.

A 100 µl reaction mix was prepared comprising the following reagents: 100 mM of deoxynucleotide mix; 80 µl sterile Hypersolv™ water (BDH); 10 pM each of forward (pAf) and reverse (pHr) primer; 10 µl 10x buffer; template DNA, equivalent to 4-10 ng environmental DNA (or 1 ng standard control pure culture DNA) and 2U Super Taq polymerase. The reaction mix was overlaid with 2-3 drops of sterile mineral oil, to prevent evaporation. To enhance product yield for environmental samples, a 'hot start' PCR protocol was adopted. Template DNA was added to the standard PCR reaction mix, the reaction was heated to 95°C for 5 minutes and then at 80°C for the addition of 2U Super Taq polymerase through the overlaying mineral oil. PCR cycling was performed using a Perkin Elmer 480 thermal cycler. The reaction parameters were 35 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes, and a final extension of 5 minutes at 72°C. Amplification products were resolved by electrophoresis of 10µl aliquots of the reaction mixtures on a 0.8% (w/v) agarose gel run in 1xTAE buffer (40mM Tris-acetate, 1mM EDTA).

Fungal PCR amplification protocol

The reagents and procedure used were similar to that for the Eubacterial PCR, but using primers specific to the 18S small nuclear rDNA region of the fungal genome. The NS1 forward primer (5`-GTAGTCATATGCTTGTCTC) and the NS8 reverse primer (5`TCCGCAGGTTCACCTACGGA) were chosen as they amplify nearly the whole of the 18S rRNA gene [6,7]. These primers were used in a 'hot start' PCR as for the Eubacterial amplification, with 35 cycles of the following conditions of 95°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes.

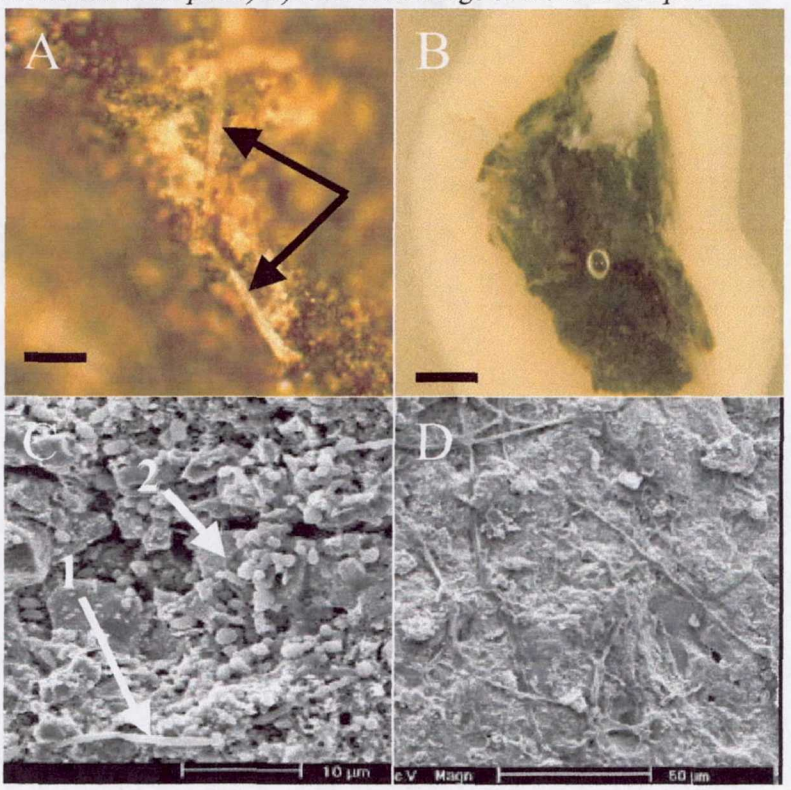
Archaea amplification PCR protocol

The reagents and procedure used were similar to that for the Eubacterial PCR, but using the forward primer (1Af) of [8] (5'-TCYGKTTGATCCYGSCRGAG) and the reverse primer (1404r) of [9] (5`-CGGTGTGTGCAAGGRGC3). A 'hot start' PCR reaction was performed as for the eubacterial PCR with the 35 cycles of the following cycling conditions as follows: 95°C, 1 minute, 50°C, 1 minute, 72°C, 2 minutes.

Results

Figure 1a shows a light microscopy image of filamentous hyphae (arrows) protruding from what appears to be a brownish secondary replacement mineral (as yet uncharacterized) on the surface of Mu 1. The filament is approximately 50 μm long and 1 - 2 μm in diameter and is in the size range characteristic of terrestrial fungi. Figure 1b is a light microscopy image showing the underside of Mu1 during the culturing experiment, white 'tracks' of bacterial growth can be seen spreading from the underside of the chip to the external surface. The outside of the chip is totally surrounded by the same white colony. Figure 1c is an SEM image of the Murchison chip after culturing showing the presence of lysed bacterial cells, bacterial spores (2) and fungal hyphae (1). Figure 1d shows an SEM image of the presence of a network of hyphael structures in the size range for terrestrial fungi on Mu2 (the sample previously investigated by D.S. McKay), with what appears to be associated exopolymeric substances (EPS) spread across the surface. No SEM images of the Allende sample are included although the enrichment culture showed the most obvious signs of colonizing bacteria.

Figure 1. All images are of the Murchison samples A) is a light microscopy image of the Mu1 sample (scale bar approx - 25 μm). Arrows show a hyphael structure. B) is a light microscopy image taken from beneath the Mu1 chip as it sits in culture medium (scale bar approx – 1mm). C) is an SEM image of the MU1 chip after its removal from the growth medium (1 - show a fungal hyphae, 2 - shows bacterial spores) D) is an SEM image of the Mu2 sample.



The presence of bacteria and fungi in this sample were subsequently confirmed by culturing experiments. Table 1 shows the results of the identification (by Gram stain, API and 16S rDNA analysis) results for a number of colonies isolated during culturing of both the Murchison and Allende samples. The bacterial species from figure 1b (the white colony around the sample of Mu1) was identified as a *Bacillus spp.* The areas showing signs of hyphal growth were scraped with a sterile inoculation loop and cultured on sabaureut dextrose agar and a single fungal species

was isolated. Characterization of this species thus far has proven difficult although genetic tests (18S rDNA characterization) are currently underway. All Allende samples (ie. Aint, Aext and Aenr) showed the presence of bacteria and in all 17 separate bacterial isolates (comprising of 10 bacterial species) and 2 fungal isolates were recovered. Several *Bacillus spp.* were identified and further analysis is continuing to obtain better DNA separation and therefore species matches on these isolates.

Table 1. The results of the identification (by Gram stain, API and 16S rDNA analysis) for a number of colonies isolated during culturing of both the Murchison and Allende samples (all results shown are for a greater than 96% match to known API (for Murchison) and 16Sr DNA databases).

Meteorite	Bacteria Identified	Fungi Identified
Murchison	*Bacillus spp.*	As yet unidentified
Allende	*Bacillus spp.*	*Neurospora spp.*
	Bacillus licheniformis.	
	Bacillus pumilis	
	Bacillus subtilis	
	Corynebacterium minutissimum	
	Micrococcus luteus	*Aspergillus spp.*
	Micrococcus spp.	
	Staphylococcus capitis	
	Staphylococcus auricularis	
	Streptococcus spp.	

DNA extractions - Allende

DNA was successfully extracted from the enrichment culture, and directly from both the internal and external chips of the meteorite sample. Figure 2 shows a photograph of an electrophoresis gel visualizing the DNA extracted from the meteorite for the Aenr, Aint and Aext samples. From this

image it appears that the quantity of DNA (shown by the intensity of the band) is in the order Aenr>Aext>Aint.

Figure 2. A photographic montage of an electrophoresis gel showing DNA extracted from the meteorite for the Aenr, Aint and Aext samples. (-ve – negative control (ultrapure water) +ve – Positive control (DNA from *Pseudomonas spp*.)).

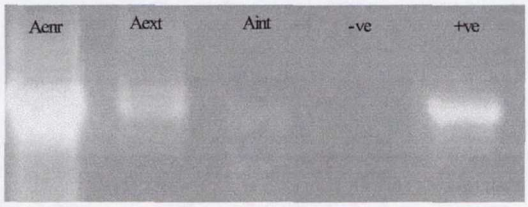
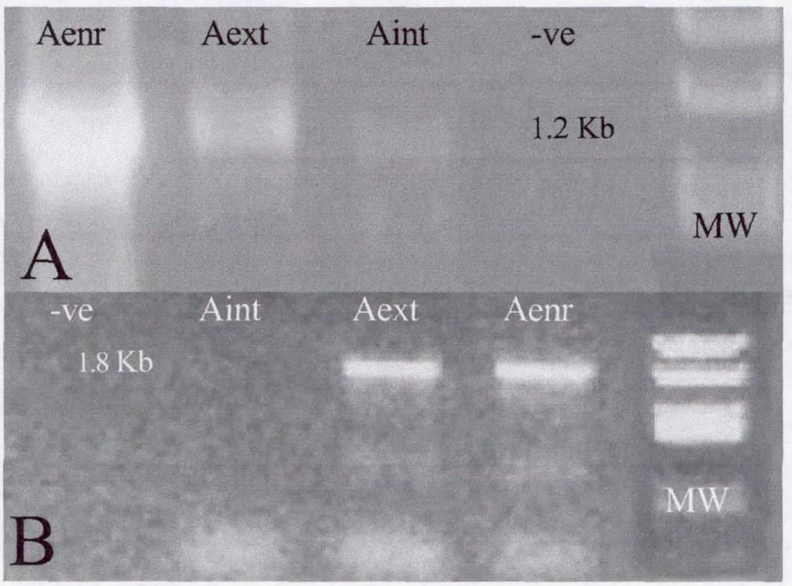


Figure 3a shows the presence of 16S rDNA PCR amplification products of the correct size (1.2 kb) indicating that Eubacterial 16S rDNA is present in all samples investigated. As expected the trend of DNA abundance closely matches that seen from the extraction gel. The PCR results as shown in Figure 3b reveal that 18S rDNA of the correct size (1.8 kb) is present in both the enrichment and external samples indicating that fungi are contaminating these two samples.

Discussion

The results presented here are not a definitive study on the contaminating microbial species from Allende and Murchison. This is because no direct DNA isolation was performed on the Murchison sample. Therefore this paper is meant to convey the evolution of the use of a molecular biology approach to microbial characterization from initial studies on Murchison, realization of the difficulties of culturing as an effective method of detailing possible microbial populations and the definition of protocols and successful isolation of DNA from Allende [10].

Figure 3. Photograph A - Agarose gel of Eubacterial PCR products (–ve: negative control (ultrapure water MW: molecular weight marker - 1.2 Kb). Photograph B - agarose gel of 18S rDNA PCR products using fungal primers (–ve: negative control MW: molecular weight marker - 1.8 Kb).



The Murchison sample showed the presence of both bacterial and fungal microbiota (Figure 1). Although the fungal species is as yet unidentified it seems reasonable to assume that both species are terrestrial. It was surprising to find that fungi had also colonized the Mu2 sample that had been previously coated (no efforts were made to isolate this fungi), this highlights the need for good curation of samples, the ease at which samples can become contaminated and that fungi can find the necessary nutrients for growth even on coated samples. Recently work conducted on Murchison has shown the presence of what has been interpreted to be *Cyanobacteria* indigenous to the meteorite [11]. Nothing resembling *Cynaobacteria* was observed from this it seems

reasonable to assume that none were present. Unfortunately no sample remained for direct DNA extraction to elucidate further whether these structures are terrestrial contamination. Fungal species do infiltrate stony surfaces and can penetrate deeply into rocks, this effect has been studied by investigators researching the biodeterioration of stone and marble and in other meteorites [12,13,14]. Although the fungal PCR in this investigation did not show the presence of fungi in the internal Allende sample. Indeed either singly or as part of lichen and cryptoendolithic communities *Cyanobacteria* themselves are known to penetrate the subsurface of rocks [15,16]. Therefore the fact that hyphael structures resembling *Cyanobacteria* have been found in the meteorite matrix is no indication of an indigenous origin. The culturing studies performed on this meteorite would automatically select for copiotrophic aerobic mesophiles and would therefore not have allowed other species such as *Cyanobacteria* to grow. This study cannot therefore present definitive proof that *Cyanbacteria* are or are not present within the meteorite. It is due to this and the fact that hundreds of possible combinations of growth medium and conditions would have to be used to ensure that all possible microbial species have been searched for, that direct DNA isolation was performed on the Allende sample.

Culturing of the Allende meteorite and subsequent sequencing yielded a number of bacterial and fungal species that are summarized in Table 1. All the bacterial and fungal species on this list are chemoorganotrophic and therefore must be gaining nutrients from the organic matter within the meteorite [17,18,19,20]. Some species are of probable human origin such as *Corynebacterium spp, Streptococcus spp*. and *Staphylococcus spp.,* interestingly *S. capitis* is found in human eyebrows and hair [17]. Members of the *Bacillus* species are all from a variety of habitats including human skin, dust and soil, this family of bacteria is extremely diverse many members are facultatively anaerobic and use both respiratory and fermentative metabolisms [17,19]. The

fungi *Neurospora* is a member of the *Ascomycetes* and is mainly found in soils, members of the *Aspergillus spp.* are ubiquitous in soils and can be associated with common laboratory contaminants and human contact [18,20]. Several of the bacteria and all the fungal species are spore forming and therefore airborne contamination is an obvious route to consider [17,20]. A further obvious source of contamination would be due to the impact of the meteorite with the Earth. None of the above organisms are exclusively found in soils and therefore no conclusions on the primary colonizers of the meteorite upon cooling can be drawn. It is difficult to form a comprehensive picture of all the microbial metabolic processes potentially occurring in the meteorite without the sequence data. Therefore, no further conclusions upon the metabolic activities of microorganisms and their effect on meteoritic organic material can be drawn in this study.

The extractions from Allende have shown that DNA can be directly isolated from contaminant organisms within and on the surface of meteorite samples (Figure 2). Although the observations were consistent with predicted results in that the interior yielded less DNA than the exterior. It was assumed that the outer surface would have the greater amount of microbial life, due to it's exposure, handling and possibly the fact that oxygen would not be a limiting factor.

16S rDNA Eubacterial PCR amplification products of the correct size (1.2kb) were generated (Figure 3a) and are currently being analysed further using Temporal Thermal Gradient Gel Electrophoresis. This data should hopefully confirm the presence of the species identified using culturing techniques and also shed light on other microbial species present. Sequencing of the DNA from the microbial species which grew in the enrichment culture (Aenr) will allow direct comparisons to those species found in the external and internal samples that received no

enrichment. This is important, as it will allow the bias imposed on the selection of microbial species when grown in defined organic rich aerobic conditions to be more fully elucidated.

The fungal PCR confirmed the results during culturing and again although no sequence data is yet available the identities of at least the two fungal species cultured are expected (Figure 3b). The lack of any fungal PCR product from the internal surface of Allende does not exclude the presence of fungi, as the DNA yield in this sample may have been prohibitively small for effective PCR [21]. Indeed it must be pointed out that these extraction techniques have been successful on a meteorite that has been curated in almost a worse case scenario and therefore these techniques must be tested on more pristine samples to attempt to fix the definitive microbial detection sensitivity of this technique in extraterrestrial samples. In general the yields of DNA from all of the samples is very low and techniques such as 'nested PCR' are being explored to increase the amplification of any extracted DNA. It may also be necessary to incorporate more cleanup steps in order to increase the quality of DNA extracted directly from the meteorite and reduce any potential inhibitors of the PCR process [21,22]. Unfortunately these results cannot yield data on the specific numbers of contaminating organisms within the meteorite as this PCR protocol was not quantitative and so future investigations will explore the use of quantitative PCR. The Archaea PCR amplification was negative and therefore no Archaeal 16S rDNA sequences were present in all the samples, which is unsurprising as it is difficult to envisage the routes of contamination of Archaea into this meteorite.

It is not surprising that either the sample of Murchison and that of Allende were contaminated given their uncertain curatorial histories. This study serves to show that correct curation is essential. All efforts were made to ensure that the samples after beginning biological investigation were only exposed to laminar flow conditions and handled in an aseptic manner with sterilized

tools. We cannot however, rule out the possibility that some of these contaminants could have been introduced during biological processing. However, if whilst using these procedures microbiota entered the samples, then it is difficult to conceive a situation where any meteorite handled will not be subjected to terrestrial microbial contamination.

That DNA can be extracted from both external and internal surfaces of carbonaceous chondrites may have major implications for the study of prebiotic synthesis of nucleotides and indeed other organics from this meteorite [23]. Especially if before the analysis took place, no effort was made to check the samples for terrestrial microbial contamination. These results are no surprise as several investigations have pointed out microbial contamination of meteorites [5,24,25,26]. However, the techniques used in this study do allow a more systematic approach to the classification of microbial contaminants and therefore to the dynamics of microbial colonization of these meteorites. Also these results show that current molecular biology techniques do not require large samples sizes to be used as effective tools for what is essentially a process of life detection in extraterrestrial materials. By studying microbial contamination of meteorites, skills needed to search for life in other parts of the solar system can be honed and new techniques polished and rigorously tested.

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