

A contamination assessment of the CI carbonaceous meteorite Orgueil using a DNA-directed approach

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[†]**In memoriam —Wilfred Röling:** On September 27, 2015, our dear colleague Wilfred Röling passed away at the age of 48. We have not only lost one of the pioneers in the area of systems biology and ecology but also a remarkable and extraordinary colleague and friend.

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Abstract—The Orgueil meteorite has become one of the most well-studied carbonaceous meteorites, after it fell in France 150 yr ago. Extraterrestrial organic compounds such as amino acids and nucleobases in the parts per billion ranges were identified in Orgueil samples with supporting isotopic analyses. However, speculations of terrestrial contamination such as organic inclusions in the form of microbes and seeds accompanied the analyses of the Orgueil meteorite ever since its fall. By using molecular analysis, we performed DNA extractions and spiking experiments combined with 16S and 18S rRNA gene targeted PCR amplification to quantify the level of terrestrial biocontamination. Our results indicate that terrestrial contamination with DNA was insignificant in the investigated meteorite fraction. We also remeasured and confirmed concentrations of amino acids found in previous studies and conclude that their rather high concentrations and distribution cannot be explained by terrestrial contamination with microorganisms alone. These results represent the first analysis using DNA-directed tools in the analysis of the Orgueil meteorite to determine trace levels of biomarkers.

INTRODUCTION

Carbonaceous chondrites contains some of the most primitive solar system materials. Their analysis opens a window for scientists to understand processes that have prevailed in the early solar nebula from which planets and small solar system bodies were formed. Composition, origin, and alteration (on the parent body) of carbonaceous chondrites have been well characterized in the last decade (e.g., Sephton et al. 2004; Alexander et al. 2007; Derenne and Robert 2010; Glavin et al. 2010; Le Guillou et al. 2012). Those meteorites contain up to 4% organic matter in soluble and insoluble form (Cronin et al. 1988). The extraterrestrial origin of many individual organic compounds has been confirmed by isotopic measurements (Engel et al. 1990; Pizzarello et al. 1991;

Engel and Macko 1997; Sephton et al. 1998, 2000). The most dominant carbon fraction (>70%) consists of insoluble macromolecular matter with an average elemental abundance of $C_{100}H_{46}N_{10}O_{15}S_{4.5}$, composed of mono- and polyaromatic units interlinked with oxygen, sulfur, and small aliphatic chains (Pizzarello and Shock 2010). A high molecular diversity of indigenous organic matter was observed for the well-studied Murchison meteorite (type CM2), using ultra high-resolution molecular analysis on the soluble fraction (Schmitt-Kopplin et al. 2010). More than 70 extraterrestrial amino acids have been identified as well as many other organic compounds, including carboxylic acids, sulfonic and phosphonic acids, and aliphatic and aromatic hydrocarbons (Cronin et al. 1993; Martins et al. 2007, 2008; Glavin and Dworkin 2009; Glavin et al. 2010). It is interesting to note that besides amino

acids, which are the monomers from which proteins are synthesized, also N-heterocycles—the basic building blocks of nucleobases in DNA—have been detected (Martins et al. 2008; Callahan et al. 2011).

The most famous carbonaceous chondrite of the CI type, Orgueil, fell in southwestern France on May 14, 1864, close to the city of Orgueil. Speculations about its organic content already surfaced in the 19th century and even Pasteur briefly examined the Orgueil meteorite (Meunier 1925). Modern, highly sensitive instrumentation and optimized analytical protocols have allowed us to characterize the mineralogical composition and organic content of the Orgueil meteorite in great detail (e.g., Gardinier et al. 2000; Ehrenfreund et al. 2001; Bland et al. 2004; Remusat et al. 2005; Martins et al. 2006; Glavin and Dworkin 2009). Amino acid analyses of pristine interior pieces of the CI chondrites, Orgueil and Ivuna, have identified only a few strong amino acid signatures, namely those of β -alanine, glycine, and γ -amino-n-butyric acid (ABA). Concentrations range from 600 to 2000 parts per billion (ppb), while most other amino acids are only present in trace levels (Ehrenfreund et al. 2001; Peeters 2007; Burton et al. 2014). The distribution and structural diversity, as well as the isotopic ratios (Ehrenfreund et al. 2001) suggest an abiotic, extraterrestrial origin for these molecules.

However, it has frequently been speculated that the Orgueil meteorite has been subjected to terrestrial contamination, which would interfere with the data interpretation of Orgueil's organic inventory. Distinct distributions of hydrocarbons in the Orgueil meteorite have been suggested to point to a biogenic extraterrestrial origin (Nagy et al. 1961). In recent years, however, isotopic measurements of “biogenic hydrocarbons” (Sephton et al. 2001), as well as evidence for terrestrial contamination of the Orgueil meteorite by hydrocarbons present in cleaning products (Watson et al. 2003), strongly suggest that at least part of the detected molecules originate from terrestrial contamination. Gounelle and Zolensky (2001) showed the presence of white sulfate veins in the Orgueil meteorite and suggested that they resulted from reactions of meteorite material with atmospheric water after the meteorite fell to Earth. Several studies have shown that the soluble organic/inorganic fraction of different types of meteorites can sustain, and even promote, microbial growth when concentrated in a soluble extract (Mautner 1997; Gonzalez-Toril et al. 2005; Gronstal et al. 2009). The Tatahouine meteorite, which was exposed for 70 yr to the environment of the Sahara desert, was shown to harbor a diverse microbial community which was also proposed to have played a role in various alteration processes detected on the surface of the Tatahouine meteorite (Benzerara et al.

2006). These results suggest that the terrestrial contamination of meteorite samples is dependent on environmental conditions at the fall site, storage, and sample handling. Already in the 1960s, Nagy and coworkers published observations on a fraction of the Orgueil meteorite regarding “organized elements” of extraterrestrial origin that supposedly resembled fossilized bacteria (Claus 1961). However, Fitch and Anders (1963) found a strong resemblance between these organized elements and common airborne pollens (most notably ragweed), arguing for a terrestrial origin of the observed elements (Fitch and Anders 1963). However, unambiguous scientifically solid proof for the presence of indigenous (fossilized) microorganisms or seeds in the Orgueil meteorite has not been put forward. Different samples of the Orgueil meteorite have most likely been subjected to different conditions and sample handling procedures in the period after its fall. It is therefore difficult to make general claims about the entire Orgueil meteorite.

This paper addresses the potential presence of terrestrial contamination in the Orgueil meteorite in the form of microbes or plant life. Amplification techniques to target and amplify specific DNA gene sequences make it possible to detect even a single terrestrial cell (Röling and Head 2005). DNA-based approaches can validate, or invalidate, claims concerning microbial contamination of meteorite samples. This paper presents for the first time such a DNA-based approach to critically evaluate the level of contamination of a fraction of the Orgueil meteorite with terrestrial organisms. We used fractions of an 18 g powdered sample from the Muséum national d'Histoire Naturelle, Paris, and conducted DNA extractions coupled to PCR amplification to determine if amplifiable terrestrial DNA was present. The amino acid content was also measured and compared to previous studies (Ehrenfreund et al. 2001; Peeters 2007; Glavin et al. 2010) as a means to monitor potential changes in the distribution over time, which could point toward microbial activity in the Orgueil meteorite. The amino acid content is discussed in relation to the results of the DNA analysis.

MATERIAL AND METHODS

Samples

The Orgueil meteorite fraction was provided by the Muséum national d'Histoire Naturelle, Paris and crushed into fine powder using a heat sterilized (500 °C, 3 h) mortar and pestle. To determine whether our method allowed us to detect seeds and pollen (especially ragweed), a variety of plant specimens including

ragweed were collected on the campus of the Vrije Universiteit, Amsterdam. Genomic DNA used for the spiking experiments was isolated from concentrated lab cultures of *Shewanella putrefaciens*.

DNA Extraction

DNA was extracted from 250 mg (for community fingerprinting) or duplicates of 125 mg (for quantification of bacterial DNA and DNA-spike recoveries) of powdered Orgueil meteorite samples. The MOBIO PowerSoil extraction kit (MOBIO Laboratories Inc., Carlsbad, CA, USA) was used for the DNA extraction of meteoritic samples. For this, the DNA extraction protocol was adapted according to Direito et al. (2012, 2014) to minimize sorption of extracted DNA by the mineral matrix. Positive controls (containing only *Shewanella putrefaciens* genomic DNA) to test for successful DNA extraction were incorporated as well as negative controls (extraction blanks with DNase- and RNase-free water [Promega, Madison, WI, USA]) to assess possible contamination during the extraction process. For the plant samples, pollen was obtained by the use of sterile synthetic cotton buds and the seeds were picked with sterile tweezers. DNA extraction from the seeds and pollen were done according to the manufacturer's (MOBIO) standard protocol.

Spike-Recovery Experiments

Spike-recovery experiments were also conducted to test for adsorption, where the meteorite samples were spiked with *S. putrefaciens* genomic DNA solution prior to DNA extraction. An incubation period of 1 h was maintained after the spike was administered, thereafter either (1) direct DNA extraction was performed, or (2) the DNA spike was left to dry in the mineral matrix under vacuum centrifugation prior to DNA extraction. All handlings of the meteorite samples and negative controls during the extraction process were conducted in a UV3 HEPA PCR workstation (UVP, Upland, CA, USA), equipped with a HEPA filter and a UV illuminator to prevent extraneous DNA contamination as best as possible.

PCR Amplification and Microbial Community Profiling

16S rRNA genes of bacteria and archaea, and 18S rRNA genes of eukarya were targeted by polymerase chain reaction (PCR) amplification. A total volume of 25 μ L was used per PCR reaction, containing 0.4 μ M forward and reverse primers (Table 1), 0.4 mg mL⁻¹ BSA (bovine serum albumin, New England Biolabs,

Table 1. Primer pairs and PCR programs used for amplification of DNA extracts.

Target	Primers	Program
Bacteria	1. F357 + GC-R518	94 °C for 5 min, 35 cycles of 94 °C of 30 s, 54 °C for 30 s and 72 °C for 30 s, with a final elongation step at 72 °C for 7 min
Archaea	1. PRA46f-Univ907r 2. PARCH340 + GC-PARCH519r	94 °C for 4 min, 35 cycles of 94 °C for 30 s, 54 °C for 1 min and 72 °C for 1 min, with a final elongation step at 72 °C for 5 min (nested approach)
Eukarya	1. Euk1A-Euk516R+GC	94 °C for 130 s was performed, followed by 35 cycles of 94 °C for 30 s, 56 °C for 45 s and 72 °C for 130 s, with a final elongation step at 72 °C for 7 min

Leusden, the Netherlands), 12.5 μ L GoTaq Mastermix (Promega, Madison, WI, USA), 6.5 μ L DNase- and RNase-free water (Promega, Madison, WI, USA), and 3 μ L DNA extract. Negative controls for PCR were conducted using DNase- and RNase-free water (Promega, Madison, WI, USA). Positive controls for the PCR consisted of isolated DNA from *Shewanella putrefaciens* (for bacteria PCR), an environmental sample known to contain Archaea (for the archaea PCR) and *Saccharomyces cerevisiae* (for the eukarya PCR). PCR products were checked by gel electrophoresis over 1.5% agarose gels, staining with ethidium bromide, and illumination under a UV transilluminator. PCR-amplified DNA extracts of the pollen and seeds were subjected to sequencing (MacroGen Europe, Amstelveen, the Netherlands) in order to verify that our eukaryotic primer sets were capable of detecting seeds and pollen.

For bacteria, the universal primer pair F357-R518 (Muyzer et al. 1993) was employed to amplify a 0.2 kb 16S rRNA gene fragment (Table 1). As PCR product was not visible after the first round of amplification, a second round of PCR, employing the same amplification conditions, was conducted on the first round PCR products. For archaea, a nested PCR approach was used with primer pairs PRA46f-Univ907r and PARCH340 + GC-PARCH519r (Ovreas et al. 1997; Vetriani et al. 1999) (Table 1). For eukaryotes, the primer pair consisted of Euk1A-Euk516R+GC (Diez et al. 2001) and was used for two subsequent rounds of PCR amplification (Table 1).

As for bacteria false positives were observed for the “negative” controls (see Results and Discussion), DNase treatments were conducted on the PCR reaction mixture to eliminate any contaminating exogenous DNA. For DNase treatments, the PCR reaction mixture was adjusted to an end concentration containing 4.75 mM MgCl₂ and 1 mM dithioerythritol (DTE), after which dsDNase (2 U μL⁻¹, Arcticzymes, Tromsø, Norway), 0.5 U per 25 μL reaction tube was added. The PCR mixture was incubated at 37 °C for 15 min to induce DNase activity. Hereafter, the reaction mixture was incubated at 65 °C for 20 min in order to irreversibly deactivate the dsDNase. Subsequently the extracted DNA template was added and PCR was performed. Control experiments were conducted to assess whether the DNase treatments had any detrimental effects on our ability to amplify and detect endogenous DNA (see Results and Discussion for more details).

Bacterial amplifications were profiled by denaturing gradient gel electrophoresis (DGGE). Gels consisted of 8% polyacrylamide (37.5:1 acrylamide/Bis) with a denaturing gradient of 30–55%. Electrophoresis was performed in Tris-acetate-EDTA (TAE) (6.5 mM, 1 mM, 2.5 mM, respectively) buffer for 4 h at 200 V and 60 °C. The gels were stained with ethidium bromide for 1 h, illuminated under a UV transilluminator and photographed. DGGE pictures were converted and analyzed with Gel Compar II software (Applied Maths, Belgium). Similarity values were calculated by Pearson correlation and visualized by unweighted paired group method with arithmetic means (UPGMA) cluster analysis. Markers, which consisted of a mixture of 12 different bacterial 16S rRNA gene fragments (M12), were run alongside the samples on the gel in order to normalize the gels (Röling et al. 2001).

DNA Quantification of Extracts by qPCR

The extracted DNA was quantified by quantitative real-time PCR (qPCR) with a 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). A volume of 25 μL was used per PCR reaction, containing 0.45 μM forward and reverse primers F357-R518, 12.5 μL 2xDynamo HS SYBR Green qPCR mix, 0.625 μL ROX internal standard dye, 4.375 μL DNase- and RNase-free water (Promega, Madison, WI, USA), 0.5 μL BSA (bovine serum albumin, New England Biolabs, Leusden, the Netherlands), and 5 μL of undiluted DNA extract. A standard curve was created from three separately prepared dilution series of genomic DNA of *S. putrefaciens* in a range going from 1 ng μL⁻¹ to 100 fg μL⁻¹ (10⁶-10² 16S rRNA copies μL⁻¹). Negative controls (DNase- and RNase-free water) were included in triplicates for the qPCR

reaction. The qPCR program was 50 °C for 2 min, 95 °C for 15 min, 40 cycles of 95 °C for 15 s, 54 °C for 30 s, 72 °C for 30 s, followed by a melting curve program to determine the melting profile of obtained amplicons and to check for the presence of formed primer dimers at 72 °C for 10 min, 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, and 60 °C for 15 s. qPCR reactions were carried out in duplicates.

Amino Acid Analysis

Amino acid extraction was performed according to a previously described protocol (Martins et al. 2007). All used glassware and ceramics were sterilized at 500 °C for 3 h. In brief, 100 mg of an Orgueil meteorite sample, in powder form, was used to extract amino acids. Heat sterilized (500 °C, 3 h) Serpentine (Nossen, Saxony, Germany) was used as procedural blank. Samples were placed in clean furnace test tubes and high purity water added before being heated to 100 °C for 24 h. Subsequently, the liquid was separated from the solid fraction by centrifugation and dried in a vacuum concentrator. Dried samples were exposed to HCl vapor for 3 h, before being desalted on cation exchange resin columns (AGX8, 100-200 mesh, Bio-Rad Hercules, CA, USA). Amino acids were eluted from the resin columns with ammonium hydroxide and eluates were dried under vacuum again. Dried samples were dissolved in high purity grade water and subjected to derivatization with o-phthaldialdehyde/N-acetyl-L-cysteine (OPA/NAC) (Botta et al. 2002). Separation was achieved on a C18 reverse phase (250 × 4.6 mm) Kinetex 5u XB-C18 from Phenomenex (Torrance, CA, USA). UV and fluorescence detection was performed on a Shimadzu RF-10AXL (excitation wavelength at 340 nm and emission at 450 nm). Amino acid identification and quantification was carried out by integrating chromatographic peaks and comparing them with known concentrations of high purity standards of individual amino acids.

RESULTS AND DISCUSSION

Cultivation-Independent Assessment of Microbial Contamination in the Orgueil Meteorite Fraction

DNA extracts from the meteorite samples were subjected to PCR amplification to assess the presence of amplifiable bacterial, archaeal, and eukaryotic rRNA genes. Archaeal and eukaryotic products were not detected after two subsequent rounds of PCR amplification (Figs. S1A,B and S2A,B in supporting information). Due to the history of Orgueil, the capability to detect seeds and pollen seemed particularly important. For this reason, a quality control was performed, where

DNA was extracted from several seed and pollen samples. These extracts were PCR amplified using the primer pair for eukaryotes (Table 1) and products were observed for all samples on a 1.5% agarose gel (Fig. S3). The PCR products were sent out for sequencing (Macrogen Europe, Amstelveen, the Netherlands). The obtained sequences all belonged to plant species and showed high identity scores (>99%), including putative species from the family Asteraceae, housing the ragweeds (sequence data not shown). Bacterial products were observed only after two subsequent PCR programs (Table 1). However, false positives were also detected in the extraction blanks and the negative control for the PCR reaction (data not shown).

The use of universal primers allows targeting conserved rRNA genes from a large variety of species at the same time and can amplify a single gene copy by a factor from 10^6 to 10^7 with very high specificity (Saiki et al. 1988). While amplification aids detection (and identification), the downside is that exogenously introduced DNA (e.g., in reagents, or introduced during sample handling) will also be amplified, complicating interpretation (e.g., Corless et al. 2000). Contaminating bacterial DNA is frequently present in the Taq DNA Polymerase used for the PCR reactions, most likely coextracted during the manufacturer's purification process (Rand and Houck 1990; Hughes et al. 1994; Maiwald et al. 1994). Evidently, contamination by exogenously introduced DNA also complicated interpretation of our results obtained using primers targeting bacterial 16S rRNA genes in general.

Therefore, DNase treatment of the PCR mixture prior to adding the DNA extract and running the PCR reaction was applied in order to remove any potential contaminants present in the reaction mixture. DNase treatments have been shown to be effective in removing contaminating DNA while still allowing the detection of even one target gene sequence per amplification reaction (Röling and Head 2005). The DNase treatment prevented the occurrence of false positives for extraction blanks and amplification controls; however, bacterial 16S rRNA gene fragments were also not observed in the Orgueil extracts. The PCR reaction mixtures of the Orgueil extracts and controls were still subjected to denaturing gradient gel electrophoresis (DGGE) (Figure 1). *S. putrefaciens* DNA was used as positive control in the extraction, and in PCR, and all DGGE profiles of these positive controls revealed an identical pattern, indicating successful DNA extraction and amplification, without a negative impact of the DNase treatment on the DGGE banding pattern (Fig. 1: +MB, +A). The multiple bands relate to the fact that the genome of *Shewanella putrefaciens* contains more than one different 16S rRNA gene sequences. Although some very faint bands appear

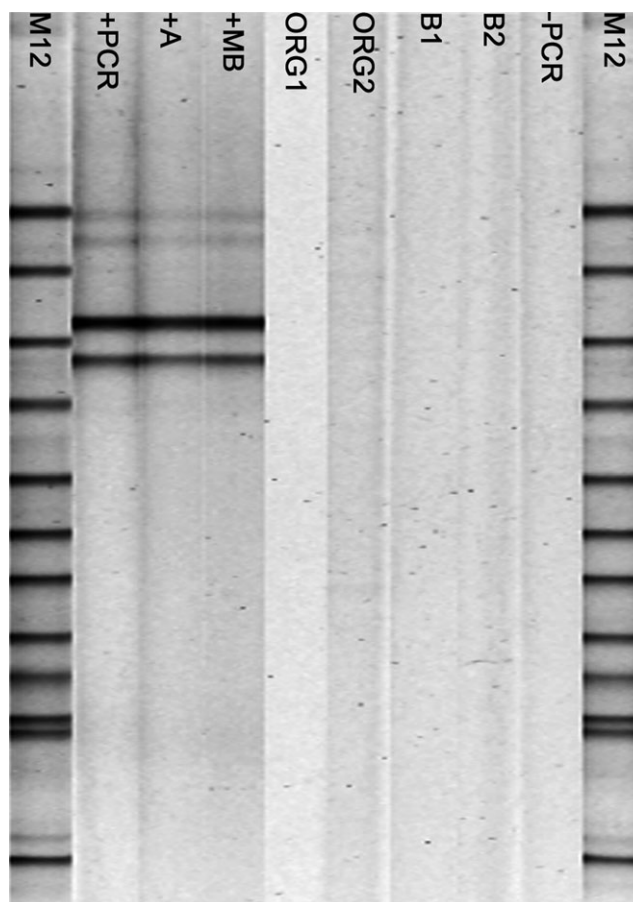


Fig. 1. DGGE profiling of bacterial 16S rRNA gene fragments, obtained using an amplification protocol that incorporated DNase treatment of the reaction mixture for all samples, except the M12 markers. *S. putrefaciens* was used for positive controls (indicated by “+”), which all show the species-specific banding pattern, while the extraction blanks and Orgueil extracts do not contain amplified product. M12 markers were run along the gel for normalization. M12 = marker; +PCR = positive control for PCR; +A = positive control for extraction with the adapted protocol; +MB = positive control for extraction with standard MOBIO protocol; ORG 1, 2 = independent Orgueil extracts; B1, 2 = independent extraction blanks; -pcr = negative control for PCR.

to be present in the ORG2 sample lane and the B2 sample lane (Fig. 1), these are not classified as such by the GELCOMPAR software, even though the sensitivity settings were set to minimal threshold. Therefore, we considered these as artifacts, or small contaminants introduced after the completion of the PCR reactions. The absence of significantly amplified product after two subsequent PCR programs in the Orgueil extracts (ORG1, ORG2, Fig. 1), as well as in procedural blanks (the extraction blanks B1, B2, and amplification blank—PCR: Fig. 1), suggests that previously observed products after amplification *without* DNase treatment were either

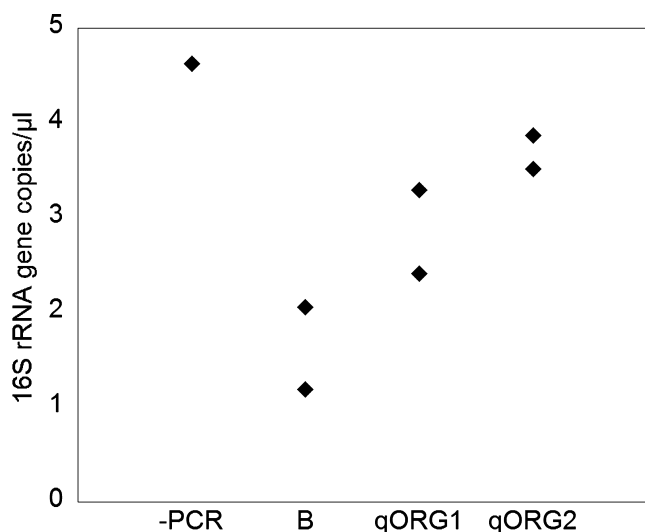


Fig. 2. Quantification by qPCR of bacterial 16S rRNA gene copies in duplicate extracts of Orgueil meteorite sample (qORG1, qORG2) including a negative control for PCR (-PCR) and extraction blanks (B).

due to (1) contamination by exogenous DNA during PCR preparation, or (2) contamination by trace levels of DNA in PCR reagents, which were successfully removed by the DNase treatment.

A qPCR experiment was performed to determine if residual DNase activity remained present despite the inactivation step prior to template addition (in the form of genomic DNA of *S. putrefaciens*) and amplification. If DNase is not fully inactivated, its continued activity may have degraded indigenous DNA present in the Orgueil meteorite extract. The quantified 16S rRNA gene copy numbers of *S. putrefaciens* were not significantly affected by DNase treatment, compared to an untreated control, neither when 500 fg DNA was used as a template in amplification ($P = 0.184$; $n = 3$), nor when 2500 pg DNA was used ($P = 0.407$; $n = 3$). Therefore, the absence of product after two subsequent rounds of PCR strongly indicates that amplifiable bacterial DNA was absent in the Orgueil meteorite extract.

Quantification by qPCR of 16S rRNA gene copy numbers in extracts derived from another Orgueil subsample revealed very low numbers, that did not differ significantly from the extraction blanks (B) and the negative control for PCR (-PCR) ($P = 0.6$) (Fig. 2). No DNase treatments were incorporated here. The very low levels of DNA in the Orgueil meteorite extracts (qORG1, qORG2) (Fig. 2) were most likely from an exogenous source because similar levels of DNA were also observed in the negative controls (B and NC in Fig. 2). These values correspond to less than three cells to a maximum of 25 cells per PCR reaction (employing 5 μ l of template), depending on the number

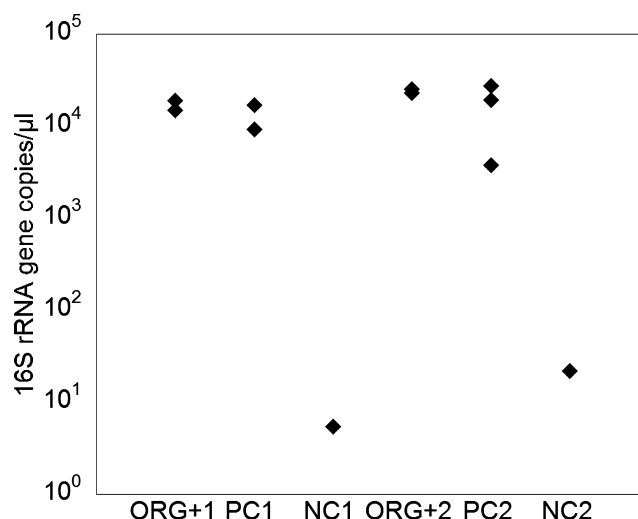


Fig. 3. qPCR quantification of recovered 16S rRNA gene copies from spiked Orgueil samples and procedural controls. Two datasets are shown. Recovery values for the “direct”-spike-recovery experiments (ORG+1, PC1, NC1) and recovery values for the “dried”-spike-recovery experiments (ORG+2, PC2, NC2). ORG+ = extract from spiked Orgueil sample $n = 2$; (PC) = procedural positive control, containing only the DNA spike $n = 2, 3$ NC = negative control, containing water ($n = 1$).

of 16S rRNA gene copies in a bacterial genome, which can vary from 1 to 15 (Vetrovsky and Baldrian 2013). All results combined suggest that no indigenous amplifiable DNA was present in the Orgueil meteorite extracts investigated here.

Spiking Experiments to Assess Adsorption of DNA and Potential PCR Inhibitors

Absence of amplifiable DNA in the extract could be due to adsorption of DNA to the meteorite mineral matrix during nucleic acid extraction. The Orgueil matrix (>60%, wt%) consists of clay minerals (saponite-serpentines) (Kerridge 1976; Tomeoka and Buseck 1988; Bland et al. 2004), which are known to hinder efficient extraction of nucleic acids (Saeki and Sakai 2009; Direito et al. 2012, 2014). Even with an adapted DNA extraction protocol, specifically aimed to minimize nucleic acid adsorption (Direito et al. 2012, 2014), losses of DNA up to 90% during the extraction process have been previously observed in the presence of certain clay minerals (Direito et al. 2012, 2014). Therefore, spiking experiments with defined quantities of *S. putrefaciens* genomic DNA were conducted, in order to test the nucleic acid recovery from the meteorite matrix.

The recovery of spiked DNA from Orgueil samples, using the adapted protocol (Fig. 3: ORG+1), was comparable to the procedural positive controls (without meteorite material, containing only *Shewanella* DNA)

Table 2. Amino acid content of analyzed Orgueil meteorite samples. Amino acid concentrations are expressed in parts per billion (ppb) and molar concentrations as determined in this study, and are compared to previous studies (Ehrenfreund et al. 2001; Peeters, 2007; Glavin et al. 2010). Standard deviations of the mean are given between parentheses.

Amino acid	Ehrenfreund et al. (2001) ($n = >2$)		Peeters (2007) ($n = 7$)		Glavin et al. (2010) ($n = >4$)		This study ($n = 1$)	
	ppb	nmol g ⁻¹	ppb	nmol g ⁻¹	ppb	nmol g ⁻¹	ppb	nmol g ⁻¹
L-Asp	54 (18)	0.41 (0.14)	147 (56)	1.1 (0.42)	55 (28)	0.41 (0.21)	104	0.78
D-Asp	28 (16)	0.21 (0.12)	52 (10)	0.39 (0.08)	54 (31)	0.41 (0.23)	79	0.59
L-Glu	61 (31)	0.1 (0.04)	99 (31)	0.67 (0.21)	83 (22)	0.56 (0.15)	117	0.8
D-Glu	15 (6)	0.41 (0.21)	107 (36)	0.73 (0.25)	47 (16)	0.32 (0.11)	40	0.27
D,L-Ser	<10	<0.095	619 (58)	5.89 (0.56)	<1	<0.01	512	4.87
Gly	707 (80)	9.42 (1.07)	524 (66)	6.98 (0.88)	865 (450)	11.52 (5.99)	310	4.13
β -Ala	2051 (311)	23.02 (3.49)	2336 (71)	26.22 (0.80)	2732 (675)	30.67 (7.58)	2262	25.39
γ -ABA	628 (294)	6.09 (2.85)	280 (21)	2.72 (0.20)	281 (131)	2.72 (1.27)	311	3.02
D,L- β -AIB	148 (70)	1.44 (0.68)	333 (24)	3.23 (0.23)	n.a.	n.a.	528	5.12
L-Ala	69 (9)	0.77 (0.10)	284 (44)	3.19 (0.49)	94 (22)	1.1 (0.25)	219	2.45
D-Ala	69 (9)	0.77 (0.10)	183 (19)	2.05 (0.212)	80 (17)	0.90 (0.19)	112	1.26
D,L- β -ABA	332 (99)	3.22 (0.96)	497 (19)	4.82 (0.18)	402 ^a	3.90 (1.64)	298	2.89
α -AIB	39 (37)	0.38 (0.36)	242 (16)	2.35 (0.16)	343 (140)	3.33 (1.36)	186	1.8

^aD,L-values were determined individually (D- β -ABA = 221 [110]; L- β -ABA = 181 [59]).

(Fig. 3: PC1), indicating successful DNA extraction. Also when the DNA spike was dried in the meteorite matrix, the recovery of the spike (Fig. 3: ORG+2) was similar to the procedural positive control (without meteorite material, containing only *Shewanella* DNA, undergoing the same procedures as the meteorite sample) (Fig. 3: PC2). These results show that adsorption of DNA by the meteorite is negligible, even if the DNA was dried in the mineral matrix. DNA extracted from spiked Orgueil samples was amplified with the same efficiency as the control indicating that PCR inhibition by potentially coextracted compounds was insignificant.

Amino Acid Concentrations in Relation to Terrestrial Contamination with Microorganisms

Amino acid concentrations in the Orgueil meteorite were determined for the first time in 2001, on a pristine interior fraction of the meteorite, and have also been conducted in later years (Ehrenfreund et al. 2001; Peeters 2007; Glavin et al. 2010). We repeated the amino acid analysis, to establish whether concentrations change over time. Our amino acid analysis of the Orgueil meteorite in 2014 revealed concentrations similar to these previous studies (Table 2). Glycine, alanine, and serine were the most notable proteinogenic amino acids detected with concentrations up to several hundred parts per billion. Glutamic and aspartic acid were also detected at slightly lower concentrations. Several non-proteinogenic amino acids revealed high concentrations, of which β -alanine was consistently the most abundant with more than 2000 ppb. Serine concentration levels in the hundreds of parts per billion range were only assigned by HPLC-FD

measurements, where unambiguous identification via the mass of amino acid derivatives is not possible. Amino acid analysis of Orgueil with mass spectrometric methods suggested concentrations of serine in the sub-ppb range (Ehrenfreund et al. 2001; Glavin et al. 2010). This discrepancy between the different studies thus appears to be dependent on the identification techniques used rather than indicating fluctuations in the concentration of serine.

Interestingly, the observed amino acid distribution shows strong similarities to amino acid contributions seen in sedimentary layers of Earth's oceans where a steep rise in dissolved glycine, β -alanine, and γ -amino butyric acid (γ -ABA) can be observed with increasing depth, while most other proteinogenic amino acids decrease or remain stable in concentration (Cole and Lee 1986; Davis and Benner 2005). Amino acid-selective diagenetic processes in sediments have been suggested to induce such specific distributions where non-proteinogenic amino acid concentrations can be used as a measure of diagenesis (Cowie and Hedges 1992; Dauwe et al. 1999). Abiotic synthesis of amino acids under prebiotic conditions has also been shown to generate relatively high amounts of glycine, alanine, and serine from basic carbon, nitrogen, and hydrogen compounds (Jiang et al. 2014). Alteration and diagenetic processes on the parent body of Orgueil may have induced similar distributions, changing the original organic inventory (Glavin et al. 2010). Thus, a variety of processes could have shaped the amino acid signature observed in the Orgueil meteorite. However, the observed relative consistency in amino acid abundances between multiple studies conducted over a period of time of almost 15 yr suggests that the amino acid content was

fairly stable in the recent past. This indicates that significant microbial consumption or alteration of amino acids for microbial growth and maintenance on the meteorite did not occur, and is well in line with the results from the DNA-directed analyses.

The observed low D/L ratios agree for a large part with previous measurements (Ehrenfreund et al. 2001; Peeters 2007; Glavin et al. 2010) (Table 2). Variations in D/L ratios however were observed for glutamic acid, which are in our measurements low when compared to the other experimental data shown in Table 2, and although Glavin et al. (2010) and Ehrenfreund et al. (2001) also detect a significant excess of the L-isomer, Peeters (2007) does not (Table 2). In contrast, the D/L ratio we determined for aspartic acid is higher than the ratios determined by Ehrenfreund et al. (2001) and Peeters (2007), while Glavin et al. (2010) do not detect a significant difference between the D- and L-enantiomers (Table 2). In the case of alanine, our results match with the results of Peeters (2007), while the results of Ehrenfreund et al. (2001) and Glavin et al. (2010) do not suggest any significant difference between the two enantiomers (Table 2). Considering the different analytical techniques and instrumentation used in the various studies, small differences in D/L ratios are not surprising. Statistical robustness due to amount and number of replica measured in the compared analyses has to be also taken into account when interpreting D/L ratio variations. Furthermore, sample heterogeneity is an important factor to consider when analyzing fractions of the Orgueil meteorite. And although we cannot fully exclude the possibility of trace contamination introduced during sample handling, extraction, and analysis, the overall distribution and levels of amino acids measured across the various studies over a time period of almost 15 yr is supporting our interpretation of no microbial contamination in the meteorite fraction analyzed.

Finally, we determined if the low amounts of cells (deduced from the quantification of extracted DNA, without including a DNase treatment step in the qPCR [Fig. 2]) in the Orgueil extracts, could theoretically explain the reported concentrations of amino acids. We estimated an expected concentration of amino acids per gram of meteorite, based on the detected 16S rRNA gene copies. *Escherichia coli*, which has seven 16S rRNA gene copy numbers in its genome (Farrelly et al. 1995), was used as a model organism for the calculations. 0.2 pg of protein per cell was assumed (Friesen 1988) and an average molecular weight of 119, based on 20 proteinogenic amino acids (in peptide chain), was used. The amount of 16S rRNA gene copies detected by qPCR (Fig. 2) corresponded to approximately 0.6 picomol individual amino acid per gram of meteorite. This value is three to four orders of magnitude lower than the

measured concentrations of individual amino acid (Table 2), suggesting that the amino acid concentrations present in Orgueil are far too high to be explained by terrestrial contamination with microorganisms alone.

CONCLUSIONS AND FUTURE PERSPECTIVES

Our results show no evidence for significant contamination of the investigated fraction of the Orgueil meteorite with microorganisms, seeds, pollen, or plants. Bacterial, archaeal, or eukaryotic DNA was not detected. Control experiments indicated that the inability to detect microorganisms did not relate to methodological biases. More importantly, terrestrial microorganisms or plant life cannot be an important source for the amino acids and their D/L ratios observed in the Orgueil meteorite.

This research was designed to address contamination by terrestrial organisms, not the presence or absence of extraterrestrial life, for which an rRNA gene sequencing approach would not be successful. Analyses of extraterrestrial material that combine different techniques such as the one presented here will be useful for astrobiology-related studies, where terrestrial contamination can potentially interfere with data interpretation. Previous studies investigating in-depth the abundance and enantiomeric ratios of amino acids in meteorites (Glavin et al. 2012; Pizzarello et al. 2012) also found enantiomeric excesses for a variety of indigenous (proteinogenic) amino acids, by using a combined approach of different techniques. By including DNA-based techniques when analyzing samples originating from outside our biosphere, one can even further exclude terrestrial life as a source of contamination, including other biomarkers not exhibiting chiral behavior. By homogenizing a small sample, it can be divided into subfractions for complementary analyses (e.g., amino acid analysis and DNA extraction). Applications of DNA-based contamination assessments can also be useful for future sample return missions in order to assess potential terrestrial contamination over time at various stages of the collection, curation, and sample analysis process.

Whole genome amplification (WGA) techniques have been suggested for the detection of extraterrestrial DNA-resembling molecules, because such techniques are not dependent on prior knowledge of DNA sequences and are highly sensitive (Direito et al. 2014). Polymerases have already been engineered to replicate molecules alternative to RNA and DNA (Pinheiro and Holliger 2012; Pinheiro et al. 2012). This may also be possible for the enzymes used in WGA, increasing the chances of detecting such, for now hypothetical molecules. Our conclusion that terrestrial DNA was not

detectable does not imply that previously observed “cellular structures” or “organized elements” are of extraterrestrial origin (Claus 1961). More molecular research needs to be performed on those structures to determine their composition and origin.

Studies showing that microorganisms can grow on the soluble organic/inorganic fraction of a variety of meteorites (including the famous Murchison meteorite) (Mautner 1997; Gonzalez-Toril et al. 2005; Benzerara et al. 2006; Gronstal et al. 2009) make it increasingly clear that microbial contamination can potentially interfere with the interpretation of the data derived from biomarker analysis of meteorites. Isotopic ratios of organic molecules or D- and L-excesses of amino acids are informative for the bulk content of meteorite fractions but are less informative in affirming findings on singular cellular structures of unknown origin.

The implementation of DNA-directed quality control for terrestrial contamination of meteorites and other extraterrestrial samples is a valuable tool in the analysis of biomarkers. The sensitivity is so high that a single copy of DNA can be detected (Saiki et al. 1988; Röling and Head 2005), which makes it an ideal technique to screen low biomass samples. Our choice to direct amplification toward relatively small DNA fragments was mainly based on the observation that partially degraded DNA often consists of short fragments of 100–500 base pairs (bp) (Paabo 1989). Besides, a further advantage of targeting a short DNA sequence is that it does not impair detecting larger DNA sequences as well. The relative short time span (~150 yr) since its fall and “safe” storage conditions of a large part of Orgueil meteorite fractions in museums or laboratories would likely not promote diagenesis of amino acids (Bada 1991) or strong degradation of DNA (Lindahl 1993; Gorbushina et al. 2007). Nevertheless, we cannot exclude alteration processes due to exposure to the environment (air, light, humidity, temperature), which could have also affected potentially present DNA. It is therefore prudent to consider DNA fragmentation and include the search for short DNA sequences when assessing contamination levels.

However, great care must be taken not to contaminate the PCR mixtures with external DNA since this will also be amplified to the same extent. DNase treatments of PCR mixtures can minimize contamination risks while including extraction blanks and procedural negative controls allows for monitoring potential introduction of contaminants. When amplification is observed, it is important to sequence the PCR products. In recognition of contamination issues in DNA-based research as applied in the field of microbial ecology, a low-biomass contamination database has been established which contains 16S rRNA gene sequences of

species that have been identified as common laboratory contaminants (Barton et al. 2006). A multidisciplinary research approach that includes critical assessment of contamination risks is the most efficient way to increase our knowledge on the organic inventory of carbonaceous meteorites and its true origin.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Fig. S1A: 1st PCR reaction products for archaea obtained using primer pair PRA46f-Univ907r

Fig. S1B: 3rd PCR reaction products for archaea obtained using primer pair PARCH340+GC-

PARCH519r to amplify the PCR product from the nested PCR reaction (2nd PCR gel not shown).

Fig. S2A: 1st PCR reaction products for eukaryotes obtained using primer pair Euk1A-Euk516R+GC.

Fig. S2B: 1st PCR reaction products for eukaryotes obtained using primer pair Euk1A-Euk516R+GC.

Fig. S3: PCR reaction products of the seed and pollen DNA extracts amplified by primer pair Euk1A-Euk516R.
