Popular Summary

A New Method for Estimating Bacterial Abundances in Natural Samples using Sublimation

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Abstract. We have developed a new method based on the sublimation of adenine from *Escherichia coli* to estimate bacterial cell counts in natural samples. To demonstrate this technique, several types of natural samples including beach sand, seawater, deep-sea sediment, and two soil samples from the Atacama Desert were heated to a temperature of 500°C for several seconds under reduced pressure. The sublimate was collected on a cold finger and the amount of adenine released from the samples then determined by high performance liquid chromatography (HPLC) with UV absorbance detection. Based on the total amount of adenine recovered from DNA and RNA in these samples, we estimated bacterial cell counts ranging from ~ 10^5 to 10^9 *E. coli* cell equivalents per gram (ECE/g). For most of these samples, the sublimation based cell counts were in agreement with total bacterial counts obtained by traditional DAPI staining.

INTRODUCTION

Direct counting (DC) methods using DNA-specific fluorochromes such as 3,6-bis(dimethylamino)acridinium chloride (acridine orange, AO) and 4,6-diamidino-2-phenylindole (DAPI) have been traditionally used to count total microbial cells in aquatic samples (9,17). DAPI specifically binds to double stranded DNA emitting a blue fluorescence when excited by 365 nm UV light, while AO-stained single stranded DNA and RNA emit an orange-red fluorescence with an excitation maximum of approximately 470 nm, enabling nucleoid containing cells (NuCC) to be identified (11). The DAPI method has been rapidly replacing AO as the bacterial stain of choice for a wide range of sample types (11), and is particularly useful for quantifying the total number of non-viable and viable but nonculturable bacteria in natural samples. However, DAPI does not stain bacteria with intact cell membranes that do not contain a visible nucleoid region (non-NuCC) and is less specific for DNA than previously thought (11,22). Moreover, the enumeration of bacteria in soils and sediments using this method can be difficult due to high background fluorescence and nonspecific staining (11). It is also important to note that any non-stainable organic compounds present in the soil and sediment derived from the degradation of living bacteria cells,

such as nucleobases or amino acids, would not be detected by these staining methods.

In previous work, we investigated a method to isolate amino acids from *E. coli* (8) and other natural samples (6) using a sublimation pyrolysis technique. In this technique, the samples were heated to ~500°C under reduced pressure. The sublimate was collected on a cold finger and analyzed for volatile amines using high performance liquid chromatography (HPLC). Although amino acids are the single most abundant compounds in *E. coli* cells, comprising 55% of the total dry cell weight (16), we found that most of the amino acids (~ 99%) originally present in the bacterial cells were destroyed during heating (8). Even though nucleic acids are less abundant than amino acids, accounting for only 24% of the dry *E. coli* cell weight (16), purines and pyrimidines are much more resistant to thermal degradation than amino acids and readily sublime directly from native *E. coli* DNA and RNA when the cells are heated (7). We found that the recovery of adenine from DNA and RNA in *E. coli* after sublimation (~99 to 100% of the theoretical value) was much higher than the other nucleobases (7), therefore adenine was used as a proxy for estimating the bacterial nucleic acid content in natural samples.

In the present study we describe a new counting method based on the sublimation of adenine from *E. coli* to estimate total bacterial abundances in natural samples. In this technique, total cell counts measured in *E. coli* cell equivalents (ECE) can be determined by a measurement of the amount of adenine sublimed from intact or degraded bacterial DNA and RNA in the samples after heating. In contrast to the fluorescent staining methods that only count cells containing discrete bundles of intact DNA, the sublimation method also accounts for intact or degraded nucleic acids from dead cells. Therefore, the sublimation counting method could be particularly useful for determining original total bacterial cell abundances in older samples containing mostly dead cells and degraded organic matter. It has recently been shown that dead cells represent the largest percentage (~70%) of total bacterial cells (dead + living cells) in coastal marine sediments (13).

MATERIALS AND METHODS

***E. coli* sample preparation and cell count determination.** All glassware used in the analyses was annealed at 500°C overnight. A crushed serpentine (hydrated magnesium silicate) sample that had been heated at 500°C for 3 h, was inoculated with *Escherichia coli* bacteria using the following procedure. *E. coli* cells (strain MG1655) were grown in glass

tubes with 500 mg of serpentine by shaking at 250 rpm overnight in 10 ml Luria-Bertani (LB) medium (21) at 37°C in a water bath. After overnight growth, a 0.5 ml aliquot of the LB medium was diluted 1/10x in culture medium, transferred into a quartz cuvette (1 cm path length), and the optical density at 460 nm (OD_{460}) then measured using a HP 8452A diode array spectrophotometer. The *E. coli* cell concentration in the medium was calculated from the OD reading using an extinction coefficient of 10^8 cells per OD_{460} absorption unit (16). The remaining LB growth medium was centrifuged for 10 min at 5000 rpm in a Falcon plastic tube to pellet the cells. The medium was decanted from the tube and the bacteria/serpentine sample re-suspended in 1.5 ml potassium phosphate buffered saline (KPBS), centrifuged at 6000 rpm for 2 min, and the supernatant removed. The KPBS washing procedure was repeated 3 more times in order to completely remove the LB medium from the sample. After rinsing, a thin bacterial film coating the top of the serpentine sample was homogenized by mixing using a sterile spatula. The inoculated serpentine sample was transferred into a capped Eppendorf vial and stored at 4°C. A crushed serpentine control blank that had not been inoculated with *E. coli* cells was also carried through the LB medium treatment, KPBS washing and mixing procedure described above.

The inoculated serpentine sample was dried under vacuum and then split into two fractions. One half of the sample was transferred to a test tube and hydrolyzed in 1 ml of double-distilled 6 M HCl at 100°C for 24 h. The HCl supernatant was then removed from the serpentine, transferred to a new test tube and dried under vacuum. The dried residue was then desalted by using cation exchange resin (AG50W-X8) and analyzed by *o*-phthaldialdehyde/N-acetyl-L-cysteine (OPA/NAC) derivatization and HPLC separation with fluorescence detection (24) in order to determine the total hydrolyzable amino acid content of the cells. Since the total concentration of protein amino acids in *E. coli* is 1.55×10^{-13} g/cell (16), an estimate of the total *E. coli* cell concentration in the inoculated serpentine can be calculated from the amino acid yield (8). The serpentine control blank was also carried through the entire procedure in parallel.

Natural samples and cell enumeration using the DAPI staining method. Several natural samples containing a wide range of bacterial cell concentrations were used in this study. A seafloor sediment at the Nile Delta from a core collected in 1964 from the German research vessel *Meteor* at a water depth of 120 m (5) was dried under vacuum and weighed. This sample was originally taken from the top 0 to 15 cm of the core and has been stored at the GEOMAR Research Center for Marine Geosciences (Kiel,

Germany) at a temperature of 10°C to 15°C from 1989 to 1999 (J. Gruetzner, personal communication). For the last 5 years, a dry sample from the core has been kept at room temperature at the Scripps Institution of Oceanography. In addition, a sample of wet beach sand (~50 m above high tide mark) and seawater from the surf zone recently collected from La Jolla Shores in San Diego, CA were also investigated. Two soil samples from an extremely arid region of the Atacama Desert in Northern Chile (15) were also analyzed. A sample of surface fines and a subsurface sample (1 cm deep) were collected in May and October of 2003, respectively, from the Flat Top Hill Site (24°S; 70°W) approximately 100 miles south of the Yungay region (personal communication, F. Grunthaner).

The seawater, sand, soil and sediment bacteria were stained in solution with DAPI and enumerated using a standard epifluorescence microscopy technique (17). Approximately 1 mL of potassium phosphate buffered saline (KPBS) was combined with 1 g of each of the solid samples or 1 mL seawater, and 100 μL 2 % borate buffered formalin was then added to the mixture. The samples were then sonicated for 3 x 1 min at 50 % power (Fisher Scientific Sonic Dismembrator 50), centrifuged at 3000 rpm for 1 min. and the supernatant removed. 5 mL of KPBS was added to the supernatant and the entire solution filtered through a 0.2 μm pore size, 25

mm black polycarbonate membrane filter (Nucleopore) to collect the bacteria from the sample solutions. A 20 μL aliquot of DAPI stain (1 mg/mL) was added to a clean microscope slide and the drop then covered with the sample membrane filter and a 25 mm square cover slip. Cell colonies were counted under UV by using a fluorescence microscope (Olympus BX51, 100x magnification) with a DAPI fluorescence emission filter. The average cell count was determined from ten separate field counts and the cell concentration (cells per sample) then inferred from the filter and field areas.

Sublimation heating experiments. A portion of the inoculated serpentine sample (190 mg) was transferred to a quartz glass sublimation apparatus (Figure 1), sealed at ~5 torr, and heated in a tube furnace (Lindberg/Blue M Mini-Mite) at ~1100°C. For the other samples, 1 g each of the Nile Delta and Atacama samples, 5 g beach sand, and 20 ml seawater were evaporated to dryness and heated separately. A cold finger, attached to the sublimation tube, was kept at -195°C with liquid nitrogen throughout the experiments. After 30 seconds, the apparatus was removed from the furnace and the pressure inside the SA brought up to 1 atm. According to thermocouple measurements of the temperature inside the apparatus, the

samples were heated to at least 500°C for ~ 30 seconds during the experiment.

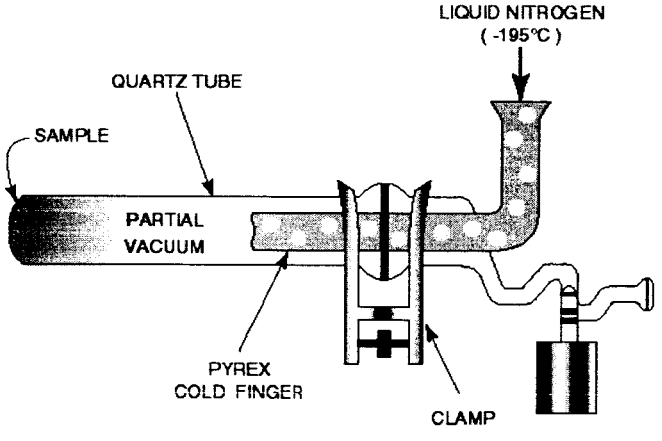

FIG. 1. The sublimation apparatus used in the heating experiments (not drawn to scale).

After sublimation of the inoculated serpentine was carried out, a yellow residue coated the end of the cold finger. This colored coating was also observed, although to a lesser extent, after sublimation of the Nile Delta, beach sand and seawater samples. We did not observe any yellow coloration of the cold finger for the Atacama Desert soils or the control blank that did not contain bacterial cells. The residue on the cold finger was carefully rinsed off in 1 ml double-distilled water and the water extract then analyzed directly by HPLC separation and UV absorbance detection. Purines and pyrimidines were separated isocratically using 0.1 M pH 4.8 sodium phosphate buffer at a flow rate of 1 ml/min on a YMC ODS-AQ reverse

phase column (4.6 x 150 mm) at 25°C using a Beckman 110B pump. Peaks were detected at 260 nm using a Kratos Spectraflow 757 UV/visible detector. Peaks were identified by comparison of the retention times with those of a standard run in parallel.

RESULTS AND DISCUSSION

***E. coli* cell counts and sublimation results from inoculated serpentine sample.** After blank correction using growth medium that did not contain *E. coli* cells, we measured $OD_{460} = 0.65$ for the *E. coli* growth medium. From the OD reading, we calculate $(6.5 \pm 0.3) \times 10^9$ *E. coli* cells in 10 ml of LB growth medium with an assumed 5% measurement error. The total number of *E. coli* cells determined from the OD reading was confirmed by a measurement of the total mass of a solid *E. coli* pellet generated by overnight growth and centrifugation of an identical volume of LB medium used to inoculate the serpentine. Assuming that the *E. coli* cells were homogenously mixed into the 0.5 g crushed serpentine sample, a concentration of 1.3×10^{10} cells/g (± 5%) for the serpentine is inferred. This value is in close agreement to a cell concentration of 1.1×10^{10} cells/g previously determined from the total amount of amino acids present in a soil analogue sample inoculated with *E. coli* after acid hydrolysis (8).

After sublimation of the inoculated serpentine sample, high concentrations (31 to 125 nmol/g) of several nucleobases, including adenine, cytosine, thymine, and uracil, were detected in a water extract of the cold finger (Table 1). Guanine did not sublime from the cells at this temperature. In a previous sublimation experiment with λDNA, we demonstrated that at a temperature of 150°C melting and fragmentation of the DNA will occur and the glycosidic bonds that attach the nucleobases to the DNA begin to break facilitating the sublimation of adenine, cytosine and thymine directly from the λDNA (7). Therefore, the nucleobases detected after sublimation of the inoculated serpentine sample were probably derived from native *E. coli* DNA and RNA. We were unable to detect any nucleobases in the UV absorption chromatogram of the control blank, which indicates that the nucleobases recovered from the inoculated serpentine were entirely derived from the *E. coli* cells and were not associated with any remnants of the growth medium used for inoculation.

From the data in Table 1, we calculate a recovery of adenine from *E. coli* in the inoculated serpentine of 125 ± 6 nmol per gram, which is equivalent to $(9.6 \pm 0.7) \times 10^{-18}$ mol adenine per cell. This value is ~10% of the theoretical yield of adenine in (16). Another possible source of adenine in *E. coli* is free ATP. However, the concentration of ATP in *E. coli* is much

lower than DNA and RNA accounting for only a small fraction (~ 0.1%) of the adenine derived from nucleic acids (16).

In order to use the sublimation recovery of adenine from *E. coli* to estimate bacterial cell equivalents in natural samples we have made several assumptions: (i) the efficiency of sublimation of adenine from *E. coli* (~10%) in serpentine is similar to natural biofilm type environments represented by the samples tested in this study, (ii) *E. coli* is representative of the types and genome sizes of bacteria present in natural samples, and (iii) all of the adenine sublimed from the samples was derived from bacterial DNA and RNA. It is important to point out that the sublimation recovery of adenine (~10% theoretical value) from the inoculated *E. coli* serpentine sample was much lower than the recovery of adenine (~99% theoretical value) directly from a solid *E. coli* pellet sublimed alone (6), indicating that the mineral matrix inhibits the sublimation of adenine from the cells. We have confirmed that the sublimation yields of a pure nucleobase standard mixture are substantially lower in the presence of serpentine. Because the inoculated serpentine sample is a closer analogue to the soil and sediment tested in this study, the recovery of adenine (~9.6×10^{-18} mol/cell) from the serpentine rather than from the *E. coli* pellet was used to estimate cell counts in the natural samples.

The wide variety of microbes and large genetic diversity of bacteria in soils, seawater and marine sediments (3, 10, 20) should be taken into consideration when using sublimation to estimate total bacterial counts in natural samples. Although the genome size of *E. coli* of ~ 4 Mbp (16) is similar to the average size of prokaryotic genomes (0.6 to 10 Mbp) as well as several common marine and soil bacteria (4), we recognize that by using *E. coli* as a reference we only obtain total bacterial counts for cells with genome sizes similar to *E. coli*. Smaller bacteria would be more abundant for the same adenine content as *E. coli*.

In addition to bacteria, there are other potential sources of adenine in the marine and soil samples analyzed in this study including phytoplankton, zooplankton, algae, plants and other debris that would contribute to our sublimation-based bacteria counts. Given these additional sources of adenine, our sublimation-based cell counts expressed as *E. coli* cell equivalents, likely overestimate the total number of bacteria present in the natural samples. Viruses are also abundant in marine seawater, soils and sediment samples with concentrations ranging from 10^6 to 10^9 free particles per ml (2, 18, 23). Given viral genome sizes of 10^4 to 10^5 bp, a significant contribution of adenine from viral DNA and RNA could have been present in the sublimed extracts of the natural samples analyzed in this study. Since

the sublimation of adenine directly from viruses was not tested, total count estimates for bacteria in natural samples using sublimation should be taken with caution. On the other hand, using the DAPI staining method, viruses would not be included at all in total bacterial counts given that they are much smaller than bacteria and single stranded viral DNA and RNA are not stained by DAPI.

Comparison of bacterial cell counts in marine samples using sublimation and DAPI. The sublimation results for the natural samples are shown in Figure 2 and Table 1. Several nucleobases including adenine, cytosine, thymine and uracil were detected on the cold finger after sublimation of the sand, seawater and Nile Delta samples. The distribution of nucleobases in these sublimed extracts was similar to the purines and pyrimidines identified after sublimation of the inoculated serpentine sample containing *E. coli* (Figure 2). We did not detect any peaks with retention times similar to those of a nucleobase standard in the HPLC chromatograms of the sublimed control serpentine blank that did not contain bacteria (Figure 2). The concentration of adenine in the sublimed extracts from these marine samples ranged from 0.005 to ~13 nmol/g. For the seawater, we calculated a cell count of 0.52×10^6 ECE/g based on the sublimation of adenine from the sample (Table 2). This cell concentration agrees reasonably well with the

DAPI cell count of 1.3 x 10^6 cells/g measured in the same sample. Both of these values are well within the range of total microbial cell counts of 0.2 to 1.6 x 10^6 cells/g obtained from seawater using whole cell fluorescent in situ hybridization (14). Our results suggest that the presence of viruses does not bias the total bacterial counts in seawater since the sublimation cell count was significantly lower and not higher than the DAPI count, which does not account for viruses. It is also possible that some adenine from dissolved cAMP present in seawater could contribute to sublimation based cell counts of the marine samples. However, given a maximum free cAMP concentration of ~35 pM for seawater (1) the contribution of adenine from cAMP in seawater would translate into a bacterial cell concentration of only 0.004 x 10^6 cells/g. This value is within the uncertainty of the sublimation based cell count of the seawater and is well below the detection limit for this method.

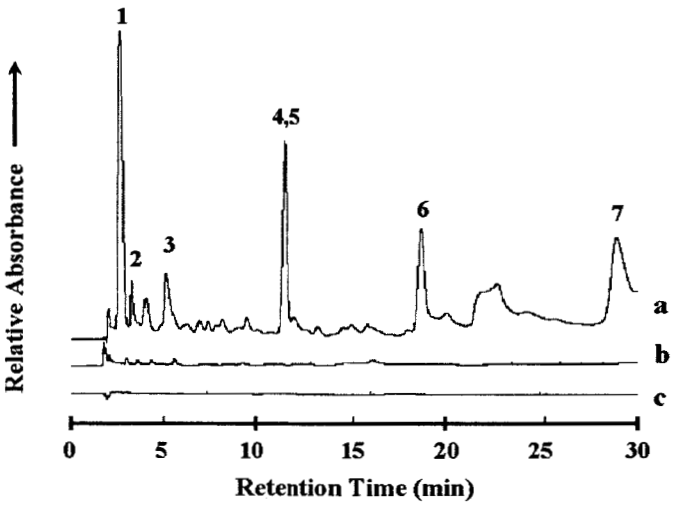

FIG. 2. The 0- to 30-min region of the reverse-phase HPLC chromatograms. UV absorbance spectra (λ = 260nm) from sublimed extracts of the Nile Delta deep-sea sediment sample (a), Atacama Desert surface fines (b), and serpentine blank (c) after heating the samples in the SA at ~500°C for 30 s. UV absorption measurements for the sublimed sand and seawater samples were also carried out, but are not shown here. Peak identifications: (1) HCl front; (2) cytosine; (3) uracil; (4,5) guanine/hypoxanthine; (6) thymine; and (7) adenine.

The concentration of sublimed adenine from the beach sand (0.2 nmol/g) and Nile Delta deep-sea sediment (12.7 nmol/g) was higher than the seawater sample (0.005 nmol/g), which is not surprising since marine sediments have much higher total bacterial concentrations than seawater per

gram. For the Nile Delta sample we calculated a cell concentration of ~1.3 x 10^9 CE/g based on sublimation. This value is within the range (0.1 to 5 x 10^9 cells/g) reported for coastal marine sediments (12, 13, 19). An estimate for the cell count based on the total hydrolyzable amino acid abundance (8) of the Nile Delta sediment sample (20.3 ppm) also yields a bacterial concentration of 0.2 x 10^9 ECE/g consistent with marine sediments. However, the DAPI staining of the Nile Delta sample gave a cell concentration of only 1.9 x 10^6 cells/g, less than 0.2 % of the ECE value obtained by sublimation (Table 2).

In most marine sediments living bacterial cells (i.e. displaying an intact membrane) account for roughly 25-30% of total bacterial counts, while dead cells represent the most abundant fraction (13). Among the living bacteria, nucleoid-containing cells (i.e. those that actively grow) represent only 4% of total bacterial counts (13). Since the DAPI method only stains NuCC, these total bacterial counts do not take into account non-NuCC dead cells and any detrital organic matter associated with them. Moreover, since the Nile Delta sediment sample analyzed in this study was from a very old core collected nearly 40 years ago and probably contains mostly dead cells and/or organic matter derived from long term degradation of cells, it is not surprising that the DAPI total cell counts were considerably lower than the values obtained

by sublimation which takes into account organic matter from both dead and living cells. Because the sublimation count of 1.3 x 10^9 ECE/g for the Nile Delta sample is consistent with DAPI counts on deep-sea sediment cores analyzed at the time of collection (13), the sublimation count may represent the total original bacterial population present in this sample. However, organic material that was not derived from bacteria in the sediment (e.g. detrital rainout from the Mediterranean Ocean surface waters) would have added to the organic content of the Nile Delta sediment sample over time. Therefore, the sublimation count must be considered to be an upper limit for the total number of cells originally present in this sample.

For the beach sand, which was collected within days of analysis, a total bacterial count of 21 x 10^6 ECE/g based on sublimation was determined (Table 2). This count was significantly lower than the Nile Delta sediment, which is in accordance with the much lower microbial load in coarse-grained sand than in mud cores such as the Nile Delta sample (12). With the DAPI method roughly 6.3 x 10^6 living cells/g was measured for the sand (Table 2, Figure 3), and is consistent with previous estimates for the percentage of living cells (~30%) of total bacterial counts in marine sediments (13).

Bacterial cell count estimates of the Atacama Desert soil. Trace levels of nucleobases (0.04 to 0.6 nmol/g) were identified in the sublimed extract of the Atacama subsurface soil sample (Table 1). Based on the amount of adenine sublimed from this sample (0.04 nmol/g), we estimate a total bacterial concentration of 4.4×10^6 ECE/g of soil. We were unable to identify any adenine in the sublimed extract of the Atacama surface fines above the ~ 0.05 nmol per gram level after heating (Figure 1, Table 1), therefore only an upper limit for the number of cells in this sample ($< 5 \times 10^6$ ECE/g) could be obtained from the sublimation method (Table 2). Using the DAPI staining method, we estimated bacterial counts for the surface and subsurface Atacama samples of $\sim 0.7 \times 10^6$ cells/g and 9.6×10^6 cells/g, respectively. These values compare reasonably well to the sublimation cell count estimates (Table 2). The DAPI counts for the Atacama soils are much higher than total viable counts of culturable heterotrophic bacteria (< 10 to 10^4 colony forming units per gram (CFU/g)) previously measured by serial dilution plating (15), which could indicate that soil samples from the Atacama Desert contain mostly non-culturable bacteria that are not detected by dilution plating. The extremely dry conditions in the Atacama Desert may inhibit biological productivity in the soils and enhance the survival of photochemically produced oxidants that

would destroy organic material (15). Although our sublimation count estimates for the Atacama soils were very near the detection limit of the analytical technique, these results confirm that the subsurface sample contains a higher organic content and bacterial cell concentration than surface fines. This may be due to the existence of a concentration gradient of soil oxidants with depth.

CONCLUSIONS

These results demonstrate the feasibility of using sublimation to estimate total bacterial counts in natural samples. Since sublimation based cell enumeration accounts for all organic matter associated with dead and living cells, this method is particularly useful for determining original cell counts or total cell equivalents in older samples that contain only low levels of viable nucleoid containing cells. In addition, the simplicity and robustness of the sublimation technique compared to the DAPI staining method makes this approach particularly attractive for use by spacecraft instrumentation. NASA is currently planning to send a lander to Mars in 2009 in order to assess whether or not organic compounds, especially those that might be associated with life, are present in Martian surface samples. The

sublimation method for determining cell counts in Martian samples is one approach that should be considered. Based on our analyses of the Atacama Desert soil samples, several million bacterial cells per gram of Martian soil should be detectable using this technique.

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Table 1. Summary of the nucleobase recovery after sublimation of several natural samples*

	Serpentine *E. coli*	Sand	Seawater	Nile Delta	Atacama Soil† *surface*	Atacama Soil† *subsurface*
Adenine	125	0.2	0.005	12.7	< 0.05	0.04
Guanine‡	nd	nd	nd	nd	< 0.05	nd
Cytosine	42	1.1	0.002	11.2	< 0.05	0.59
Thymine	31	1.0	0.010	3.0	< 0.05	0.13
Uracil	68	0.8	0.005	16.8	< 0.05	0.05

*The total recoveries of several nucleobases (nmol/g) from water extracts of a cold finger after sublimation. For the heating experiments, 0.2 g of inoculated serpentine, 5 g of sand, 20 mL or ~ 20.5 g of seawater (density assumed to be 1.027 g/ml), and 1 g each of the Nile Delta and Atacama Desert soil samples, were prepared and analyzed as discussed in the text. All reported values were corrected using the procedural blank. The uncertainty in the measurements was ~ ± 5% based on the reproducibility of standards run on the same day as the samples.

†Upper limit for the recoveries from the Atacama soil were based on the detection limit for these nucleobases using HPLC and UV absorption.

‡Guanine did not sublime at these temperatures; nd = not determined.

Table 2. Bacterial cell counts in different natural samples

Sample	Characterization	Bacterial abundance (10^6 cells/g)	
		Sublimation*	DAPI†
Seawater	La Jolla surface sample	0.52 ± 0.04	1.3 ± 0.2
Sand	La Jolla beach sand	21 ± 2	6.3 ± 1.1
Nile Delta	deep-sea core (upper 15 cm)	1320 ± 90	1.9 ± 0.3
Atacama	surface fines	< 5	0.7 ± 0.1
Atacama	subsurface (1 cm deep)	4.4 ± 0.3	9.6 ± 1.6

*Bacterial counts in *E. coli* cell equivalents (ECE) were calculated by dividing the sublimation recovery of adenine from the samples in Table 1 by an adenine yield of 9.6 x 10^{-18} mol adenine per cell from the inoculated serpentine soil analogue. Propagated errors in the cell counts were based on an uncertainty of ± 5% for the adenine yields.

†For the DAPI cell counts (cells/g) we assumed an average uncertainty of ± 17% based on measurements made in a previous study (11).