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Microorganisms cultured from stratospheric air samples obtained at 41 km

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Abstract

Samples of air removed from the stratosphere, at an altitude of 41 km, were previously found to contain viable, but non-cultureable bacteria (cocci and rods). Here, we describe experiments aimed at growing these, together with any other organisms, present in these samples. Two bacteria (*Bacillus simplex* and *Staphylococcus pasteuri*) and a single fungus, *Engyodontium album* (Limber) de Hoog were isolated from the samples. Although the possibility of contamination can never be ruled out when space-derived samples are studied on earth, we are confident that the organisms originated from the stratosphere. Possible mechanisms by which these organisms could have attained such a height are discussed.

Keywords: Exobiology; Astrobiology; Panspermia; Extreme environment

1. Introduction

The extension of the biosphere upwards to include various levels of the atmosphere has been discussed intermittently for many years, particularly in relation to the transport of pathogenic microorganisms from one part of the globe to another [1]. The occurrence of microorganisms in cumulous clouds is not in dispute, nor is their role in nucleating atmospheric ice crystals [2,3] as such organisms are likely to be of terrestrial origin.

Attempts were made to probe the stratosphere in the years immediately prior to the space age [4]. Although it was claimed that bacteria and fungi can be found over the altitude range 18–39 km, such results were generally dismissed on the basis of contamination.

Narlikar et al. [5] sought to repeat these early experiments using rigorous sterilisation protocols, combined with state-of-the-art balloon experimentation technology used in India for research in atmospheric physics as well as cosmic ray and infrared astronomy.

In an earlier paper Harris et al. [6] reported the discovery of clumps of cocci-shaped sub-micron-sized particles of overall average radius 3.0 μm from isolates of filters that were recovered from an earlier stratospheric probe. The clumps were identified, as bacteria, first using a scanning electron microscope and later using an epifluorescence microscope. The latter technique used a membrane-potential-sensitive dye (carbocyanine) and fluorescence was interpreted as revealing the presence of viable cells. Here we describe studies aimed at isolating and growing these previously viable, but non-cultureable bacteria.

2. Materials and methods

2.1. Stratosphere sampling

Air samples were collected over Hyderabad, India on 20 January 2001 at various heights up to 41 km. The collection involved the deployment of balloon-borne cryosamplers of the type described by Lal et al. [7]. The cryosampler comprised of a 16-probe manifold, each probe made of high-quality stainless steel capable of withstanding pres-

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sures in the range 10^{-6} mb (ultravacuum) to 200 b. The probes and all their components were thoroughly sterilised. They were flushed with acetone and heat sterilised to temperatures of 180°C for several hours. The entrance to each probe was fitted with a metallic, motor driven (Nupro) valve that could be opened and shut on ground telecommand. The payload trailed at a shallow angle of elevation behind the balloon gondola, being tethered by a sterilised 100-m-long rope. As a further precaution against the possibility of collecting any traces of out-gassed material from the balloon surface, a sterilised intake tube 2 m long formed an integral part of the cryosampler ensemble. The intake to each probe was covered during the balloons ascent through the atmosphere.

Throughout the flight the probes remained immersed in liquid Ne so as to create a cryopump effect, allowing ambient air to be admitted when the valves were opened. Air was collected into a sequence of probes during ascent, the highest altitude reached being 41 km. The cryosampler manifold, once the probes were filled, was parachuted back to ground. The probes were stored at −80°C until the laboratory work began.

Laboratory analysis relating to only two probes is discussed here:

1. Probe A: Collection between 30 and 39 km altitude, a total quantity amounting to 38.4 l of air at normal temperature and pressure (NTP).
2. Probe B: Collection between 40 and 41 km altitude, a total quantity amounting to 18.5 l of air at NTP.

Two procedures were used to extract aerosols aseptically from the probes:

1. Procedure 1: The air from the exit valve of each probe was passed in a sterile system in a microflow cabinet sequentially through a 0.45-μm and a 0.22-μm micropore cellulose nitrate filter (filter diameter 47 mm).
2. Procedure 2: Following the completion of Procedure 1, the probes were injected with sterile phosphate buffer solution, agitated for several hours in a shaker to dislodge particles adhered to the walls, and the liquid syringed out and passed sequentially through three filters: (i) 0.7-μm glass microfibre filter, (ii) 0.45-μm cellulose acetate filter, and (iii) 0.2-μm cellulose acetate filter.

Most of the aerosols are expected to have been collected in Procedure 1.

2.2. Microbial isolation studies

Membranes, stored at −80°C were aseptically transferred (using a laminar air flow cabinet) to sterile, plastic universal bottles, and left at ambient (15–20°C) temperature for 4 days. Sterile, distilled water (10 ml) was added to each tube and allowed to soak the membranes for 1 h. The tubes were then shaken vigorously on an orbital shaker for 5 min. Samples (0.5 ml) of the water extracts were then transferred to the surface of the following media (Oxoid, autoclaved at 120°C): Columbia base; Mueller Hinton; L Broth; nutrient agar; potato dextrose agar (PDA) and Czapek Dox. Nutrient-free silica gel was also included in an attempt to isolate oligotrophic microorganisms [8]. The extracts were spread over the surface of the medium by gentle hand motion (not by the use of spreader). The plates were then incubated at 25°C and examined periodically. Microscope studies involved the use of an optical microscope (×1000, oil emersion, phase contrast) and by scanning electron microscopy (SEM) after gold coating.

2.3. SEM

Sterile, distilled water was added to the surface of fresh, inoculated PDA plates, which were swirled gently by hand. Samples of the water extract were then aseptically removed using a sterile syringe, such that the Petri dish lid remained as close to the base as possible. The extract was transferred to a sterile membrane filter apparatus (Nalgene, 100 ml, 0.2 μm) and filtered using low suction. The membranes were then removed and transferred to a sterile Petri dish and allowed to air dry. They were then examined using SEM.

2.4. Precautions taken against contamination

Standard microbial techniques, employing a laminar air cabinet, were used throughout. All plates were used within 8 h of pouring and were checked for contamination, both by light microscope examination and by culturing. Control membranes were also analysed after being subjected to identical transfer procedures used for stratosphere-derived samples. In addition, the isolation experiments were repeated in a separate laboratory, by a technician who was not informed of any expected outcomes.

3. Results and discussion

After 4 days incubation no bacterial colonies were seen on any of the inoculated plates independent of the medium used (including nutrient-free silica gel). In order to determine if bacteria were present, but not forming colonies, the surface of the media was gently removed using a sterile scalpel and viewed under the optical microscope. Bacteria (coccus and rod forms) were seen only in surface medium removed from the PDA plates. The coccus was seen more frequently than the rod and occurred singly or in clumps of two or three cells. The rod was not seen in samples of PDA when examined under the SEM. The coccus in contrast was seen under the SEM occurring in clumps of coccoid cells (0.5–2.0 μm diameter, Fig. 1). It is noteworthy that the viable, but non cultureable, organisms seen (using SEM) by Harris, et al. [6] consisted of clumps of cocci, and an occasional rod.

Samples of surface medium obtained from undisturbed

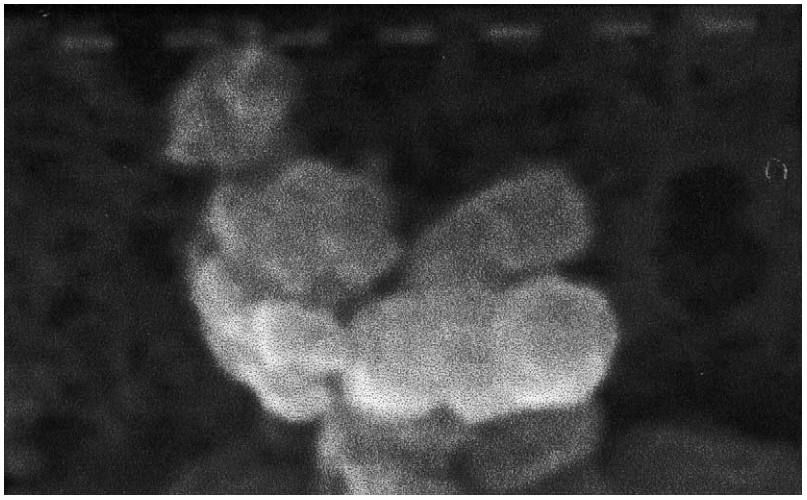

Fig. 1. SEM photograph of coccoid cells removed from surface of PDA. Bar represents 1 μm.

plates were then removed and transferred to L-broth (incubated with shaking, at 30°C for 4 days). Liquid medium was removed from any tubes showing turbidity and plated (in the standard manner) onto LB medium that was then incubated at 30°C until bacterial growth appeared. After transfer to LB medium (incubated for 2 days at 30°C) bacterial colonies appeared which comprised rod and a coccus. The rod was large and formed long chains and, after extended incubation, formed spores. The coccus organism occurred singly, in pairs or clusters. Both organisms were Gram-positive, non-acid fast, and catalase-positive, and facultatively anaerobic. The coccus was initially distinguished from *Staphylococcus* by its ability to grow on furazolidone (Sigma, 100 μg ml^{-1}), tentatively indicating it to be a species of *Micrococcus*; the rod was tentatively identified as a species of *Bacillus*. The cultures were independently identified using 16S rRNA analysis (by NCIMB, Aberdeen, using the MicroSeq™ database). The coccus and rod were identified respectively as *Staphylococcus pasteuri* (99.9% similarity) and *Bacillus simplex* (100% similarity). The *B. simplex* isolate is phylogenetically closely related to *Brevibacterium frigoritolerans* (Fig. 2a); while the *S. pasteuri* isolate, while being closely related to *Staphylococcus warneri*, is relatively phylogenetically distinct from *Staphylococcus epidermidis* (Fig. 2b). Further details of the characteristics of *B. simplex* and *S. pasteuri* are respectively given in Priest et al.[9] and Chesneau et al. [10].

No organisms were isolated using nutrient-free silica gel medium. This suggests that oligotrophs were absent or, if present, were incapable of growing under the physical–chemical conditions provided by the medium.

In addition to the two bacteria, a single fungus was isolated on the PDA plates. It was identified by CABI Bioscience (Egham) as *Engyodontium album* (Limber) de Hoog [11]. This fungus appeared on a single isolation plate; no other fungi were isolated on any other media.

Bacteria were only observed growing on, and isolated from, the surface of PDA. An element of serendipity was involved in the isolation of these bacteria since the only samples of this medium available to us at the time was approximately 15 years old, and showed signs of browning due to oxidation. When prepared, the powder produced a

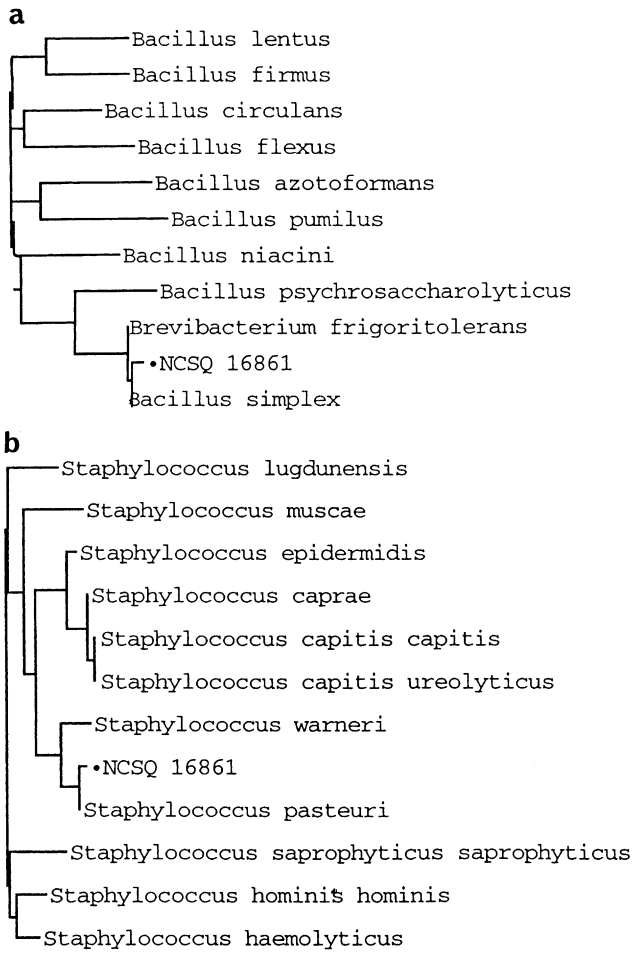

Fig. 2. Dendrograms showing phylogenetic relations of (a) the rod-shaped isolate (NCSQ 16861 and (b) the coccus (NCSQ 16861).

brown medium that set, but which was softer than both normal PDA, and the other media employed. It is possible that the oxidised, soft characteristics of this medium may have encouraged bacterial growth; it is noteworthy that soft (semi-solid) agars have been used before in bacteriology, to isolate organisms [12].

3.1. Comments on the origin of the isolated organisms

Since these organisms are found on earth it is impossible to state categorically that they are not contaminants. However, every effort was made, within the limits of our resources, to avoid contamination and to check at every stage that all materials were sterile and free from contamination. For example, none of the organisms were isolated on non-inoculated plates left exposed to the atmosphere in the laminar air-flow cabinet, or from breath plates and glove or skin washings. Similarly, no contaminants were found on any of the media, sterile water, or control membranes used in this study. A technician (who was unaware of any expected outcome and working in a separate laboratory) also repeated the isolation protocol and isolated the same two bacteria seen by us. Finally, the isolates are not common laboratory contaminants and have never been used in any of the laboratories involved in these studies. It is particularly noteworthy that none of the bacterial isolates formed colonies on any of the initial media employed, as would have been the case with air contaminants. The balance of probabilities would therefore suggest that the organisms were indeed obtained from the stratosphere.

The survival of microorganisms in the stratosphere will be particularly limited by exposure to UV light. It is generally accepted that spore-forming bacilli, such as *B. simplex* are relatively resistant to such radiation, as are bacteria whose vegetative cells tend to clump together; essentially because the outer cells provide a UV barrier that protects the inner cells. Whisler [13] found that *Sarcina lutea*, which forms UV protective packets of cells, is 100 times more resistant to UV than is *Escherichia coli*, while *B. subtilis* and *Staphylococcus aureus* are respectively three and eight times more resistant. It is not surprising therefore that our stratospheric bacterial isolates exhibit potentially UV-resistant morphologies. The environment found at 41 km will obviously be extreme, not only in terms of UV exposure, but also because of low temperatures and pressures. At first sight, it would appear unlikely that microorganisms could withstand such extremes. However, *Streptococcus mitus* has been shown to survive for 31 months on the Moon's surface, while *Bacillus subtilis* has been recovered in a viable state after 6 years of exposure to the space environment [14,15].

If, as seems likely, the microorganisms isolated here were obtained from the stratosphere, how did they get to a height of 41 km? The two obvious sources are (a) from Earth and (b) from space.

3.2. Discussion of a possible Earth origin for the isolates

It is generally accepted that few particles of Earth origin can cross the tropopause, a natural barrier occurring at around 17 km above the Earth's surface. While convection currents mix ground level particulates in the air and readily carry them into the troposphere, temperature inversions beyond 15 km lead to barriers through which very few aerosols can penetrate. Whenever rare events such as volcanic eruptions loft particles above 30 km, particles larger than a few microns fall back quickly to the ground under gravity. The isothermal temperature regime between 15 and 25 km effectively stops the ascent of particulates, and the rapidly rising ambient temperature gradient at higher levels should make the upper stratosphere almost impervious to the transport of aerosols from the ground.

A volcanic origin for the bacteria sampled in this study is ruled out for the simple reason that there was no volcanic eruption recorded in a 2-year run-up to the balloon launch date on 20 January 2001, and for reasons already stated, a settling rate at 0.18 cm s^{-1} from 41 km, as calculated by Colbeck [16] would drain out particles of 3 μm radius in a matter of weeks. A similar objection applies to rare meteorological events. Assuming our collections on 20 January 2001 gave us representative stratospheric samples at 41 km no process that is purely terrestrial can sustain the high densities of bacterial clusters as are implied, such densities would require an in-fall, or fall-back rate of a factor of 1 t $year^{-1}$ over the entire planet [6].

3.3. Discussion of a space origin for the isolates

An extraterrestrial origin of the isolates [17] provides a consistent, if controversial, explanation of our findings. The bacterial material, cultured in the present experiment, and detected earlier through fluorescence microscopy, can be regarded as forming part of the 100 t day^{-1} input of cometary material known to reach the Earth. Critics of panspermia may argue that 3 μm radius particles get burnt through frictional heating and end up as meteors. Some fractions may do, but others would not. Survival depends of many factors such as angle of entry and mode of deposition in the very high stratosphere. Several modes of entry can be considered that permit intact injection into the stratosphere, possibly starting off as larger aggregates released from comets that disintegrate into a cascade of slow-moving smaller clumps at heights above 270 km where frictional heating would be negligible. Evidence for such disintegrations has been available for many years [18], and more recent studies of particles collected using U2 aircraft have also shown the survivability of extremely fragile organic structures.

Many microbiologists will probably react negatively, as we initially did, to the suggestion that the any stratospheric bacterial flora would include species of the genus *Staphylococcus*. This view is prejudiced by the preconception

that Staphylococci are solely human pathogens; in reality however, members of this genus are hardy organisms that can exist in a variety of natural environments.

The main theoretical limitation on the view that microorganisms, such as *B. simplex*, *S. pasteuri* and *E. album*, which are found on Earth, arrive from space does not however, relate to problems concerned with survival. Instead, such a bias relates to fundamental genetic and evolutionary considerations. This is because it is considered difficult to reconcile prevailing ideas on the evolution of microorganisms and recognised phylogenetic relationships with the view that organisms, identical to those found on Earth, are continually entering the system from another source; except that is if evolution occurs elsewhere at rates and in the direction which are identical to evolution on Earth, or if terrestrial evolution is driven from outside. It is noteworthy however, that bacteria with genome sequences essentially identical to those of modern bacteria have been isolated in salt crystals of known ancient provenance [19,20]. Such studies have created considerable controversy since they, like our findings, would suggest that either the isolates were contaminants or that evolutionary and phylogenetic microbiology is more convoluted than is generally thought [21].

4. Conclusions

Studies such as these, in which space-derived samples are returned to earth for microbial sampling can readily be dismissed on the basis that any organism isolated must be Earth-based contaminants, even though the balance of evidence suggests that this is not the case in our study. Future planned cryoprobes will hopefully help to demonstrate that our results are repeatable. However, the only certain means of proving the existence of microorganisms in the stratospheres is to send probes where samples can be analysed in situ in space; hopefully the present studies will encourage the sending of such probes in the not too distant future.

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