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# Confirmation of the presence of viable but non-cultureable bacteria in the stratosphere

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**Abstract:** The presence of viable, but non-cultureable, bacteria on membranes through which stratospheric air samples were passed has been confirmed using viable fluorescent staining.

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## Introduction

The presence of viable bacteria in air sampled from the stratosphere at a height of 41 km has been previously reported (Harris *et al.* 2001). Subsequently, two bacteria (*Bacillus simplex* and *Staphylococcus pasteurii*) and a fungus (*Engyodontium album*) were independently cultured from the samples (Wainwright *et al.* 2003). These reports show that living organisms occur at a height of 41 km, i.e. well above the tropopause (17 km). Electron microscope studies have also provided tentative evidence for the presence of bacteria at a height of 25 km (Bigg 1984); however Bigg made no attempt to stain or grow these supposed bacteria.

Microorganisms present at 41 km above the Earth could have originated from Earth or space (or possibly a combination of both); the view that such microorganisms originated from space is obviously controversial. Studies on bacteria in the upper atmosphere are also relevant to the transport of animal, plant and human pathogens around the globe. Here we describe experiments aimed at confirming the presence of viable bacteria in the stratosphere.

## Materials and methods

### Collection of stratospheric samples

The stratospheric air samples were obtained from a height of (a) 30–39 km and (b) 40–41 km using a cryosampler attached to a balloon. Strenuous efforts were made to avoid contamination from the balloon, and of the sampling probe during the balloon's ascent into space, further details of which are reported elsewhere (Wainwright *et al.* 2003). The balloon carrying a cryosampler payload was launched on 21 January 2001 from Hyderabad, India. The cryogenic sampler

comprised a 16-probe assembly. Each probe had a volume of 0.35 l and was made of high vacuum grade stainless steel. It was capable of holding a vacuum at  $10^{-6}$  mb and a pressure of 600 b. The temperature cycling ability of the probes was tested between  $-246$  and  $140$  °C. To minimize contamination, only the minimum required electron-beam welds were made and the interior was electroplated. The probes and manifolds were cleaned with acetone and four washes of demineralized water; the complete assembly was steam baked and finally heated with infrared lamps to  $140$  °C. To prevent collection of out-gassed substances from the gondola, a 2 m intake tube (sterilized as above) formed a part of the payload assembly and the assembly was tethered to the balloon by a sterilized 100 m line. The probe mouth was covered during balloon ascent and had a metallic (Nupro) valve, which could be remotely operated from the ground.

### Viable staining

The air samples collected from the stratosphere were passed through 0.2 and 0.45  $\mu$ m cellulose acetate membrane filters. Some of these samples were used by Harris *et al.* (2001) to detect the presence of viable bacteria; studies on some of the remaining samples are discussed here.

Sterile distilled water (1 ml) was added to samples of portions of membrane (of varying sizes) contained in sterile plastic Universal bottles. Membranes through which air passed at both heights were used. The bottles were shaken vigorously for 15 minutes and the presence of viable microorganisms was determined by using a Live/Dead *BacLight* Bacterial Viability Kit (Molecular Probes Inc., Netherlands), as described in the maker's instructions. Two control membranes were included: (a) a sample of membrane that had been exposed to the same handling procedures as above,

but which had not been exposed to the atmospheric air sample and (b) portions of a new, unused membrane. Samples of the stained solutions were then examined under oil immersion (100x) using a fluorescent microscope (Olympus BX41, fitted with a U-RFL-T lamp, using a wide blue filter). An equal amount of time was devoted to examining both test and control samples, photographs were taken using a digital camera (Olympus C4040 200M, calibration carried out using Olympus DP-soft imaging software).

#### Culturing techniques

Portions of the membranes were shaken by hand in sterile, distilled water and Ringer's solution (quarter strength). Aliquots (0.1 ml) were then transferred to the surface of the following media (Oxoid): Czapek Dox, Nutrient Agar, LB medium, potato dextrose agar, blood agar and nutrient free silica gel (in an attempt to isolate oligotrophs). The plates were incubated aerobically at 25 and 30 °C and anaerobically at the same temperatures (in a gas jar containing an atmosphere of CO<sub>2</sub> and H<sub>2</sub>). The plates were incubated for 7 days and, at intervals, checked for the appearance of surface bacterial colonies. The presence of non-colony forming bacteria on the surface of the medium was also checked; liquid removed from the surface of the medium was examined using a light microscope (1000x, phase contrast). A laminar air-flow cabinet and standard sterile technique were used throughout.

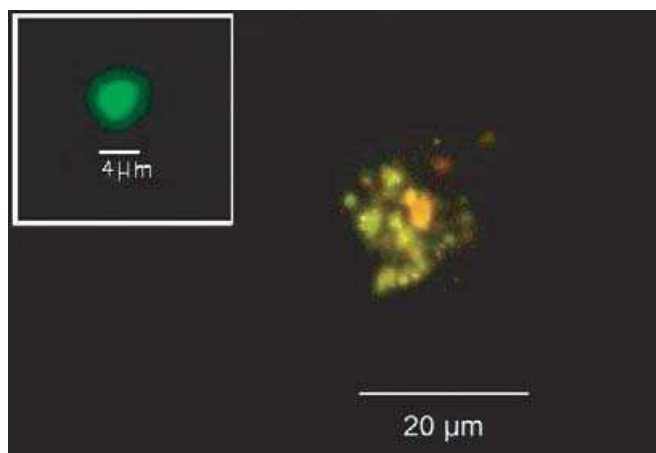
#### Results and discussion

The work reported by Harris *et al.* (2001) involved the use of the fluorescent membrane potential sensitive dye, carbo-cyanine. Using this stain, small clumps of coccoid cells of the dimension of bacteria (1–2 µm) were seen on membranes through which stratospheric air had been filtered. From these observations, Harris *et al.* (2001) concluded that the observed fluorescing particles were viable bacteria; they did not, however, comment on their cultureability.

The aim of the work described here was to attempt to provide independent confirmation of the presence of such viable bacteria in samples obtained from the stratosphere.

The Live/Dead probe used here utilizes mixtures of a green fluorescent nucleic acid stain and the red fluorescent nucleic acid stain, propidium iodide. When used alone, the green stain generally labels all bacteria, including those with intact or damaged cell membranes. In contrast, propidium iodide penetrates only bacteria with damaged membranes causing a reduction in the green stain when both dyes are present; as a result, the probe stains live cells fluorescent-green, while dead bacteria stain red.

Figure 1 shows that both green fluorescent and red staining bodies were present on the membrane filters through which stratospheric air, at both heights, had been filtered. By careful focusing of the microscope these were seen to consist of individual cocci, or clumps of a small number of coccoid-shaped cells, having both the dimensions (circa 2 µm) and appearance of bacteria. No similar, fluorescent green or red-staining bodies were seen on newly opened membranes, or



**Fig. 1.** Bright (green, living) and diffuse (red, dead) fluorescent particles, having the size and morphology of bacteria, found on membranes through which stratospheric air was passed; sampled at a height of 30–39 km and (inset) 40–41 km.

control membranes exposed to the same handling protocols. As a result, we conclude that the green fluorescent cells observed on the space-derived samples are viable bacteria, and that by using an approach to viable staining, differing from that used by Harris *et al.* (2001), we have confirmed the presence of viable bacteria in samples derived from the stratosphere.

Neither bacterial colonies nor non-colony forming bacteria were isolated from the membranes using the media and protocols employed. Within the limits of our studies, the fluorescent-staining coccoid cells, seen on the membranes, can be regarded as being viable but non-cultureable (VBNC) bacteria. Of course, cultureability depends on the type of media and conditions employed; as a result, the bacteria observed might have grown had different media and growth conditions been employed. Two bacteria (*Bacillus simplex* and *Staphylococcus pasteurii*) and a single fungus (*Engyodontium album*) have been isolated (by employing some of the media used here) from some of these stratosphere samples (Wainwright *et al.* 2003). However, the fact that viable microorganisms were only cultured on only one occasion, from a single membrane, suggests that most of the stratospheric bacteria present on the membranes are VBNC cells.

In conclusion, our results confirm that VBNC bacteria are present in the stratosphere. Clearly, further probes need to be sent to the heights sampled here before the issue of whether such bacteria originate from Earth or space can be concluded; to this end, we are currently attempting to conduct carbon isotope fractionation studies on the membranes to determine if the bacteria seen on them are of terrestrial origin.

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