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# Evidence That Bacteria Can Form New Cells in Airborne Particles

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*Serratia marcescens* incubated for 8 h at 31°C in a chemically defined medium contained in shake flasks was aerosolized into rotating-drum aerosol chambers at 30°C and saturated humidity. Cells furnished tryptone (Difco) and glycerol just before aerosolization increased (in viable numbers and countable cells) almost twofold within 1 to 2 h after becoming airborne, whereas cells not furnished additional tryptone decreased in viable numbers at a faster rate than the number of particles removed by gravitational settling. Limited tests with a Coulter Counter showed that cell volume changes occurred in growing cells that did not occur in the nongrowing population.

This paper is one of a series describing studies on whether bacteria can reproduce within small, airborne particles. Although the primary goal is to determine whether the gaseous atmosphere of Jupiter (7) might accidentally be contaminated by entry of a space probe, our interest at this time is only to determine whether airborne bacteria could reproduce under any circumstances. We had found that airborne bacterial cells could maintain metabolic functions (3) and could apparently produce new deoxyribonucleic acid (11). We mentioned, in addition, the finding of marginal evidence for cellular division.

Our approach to this problem used a common procedure in experiments in aerobiology, that is, to aerosolize a bacterial suspension into preconditioned chambers at desired temperature and humidity, sample at appropriate times and intervals, dilute (or not) the sampling fluid by 10-fold amounts, plant known volumes of sampler fluid on appropriate agar plates, and count the colonies after incubation. These data can be transformed to number of viable cells per liter of air (2).

Numbers of viable, airborne cells, as well as particles, decrease with time in the chambers because of both "death" of cells and gravitational deposition (fallout) of particles. The latter can be measured by either nephelometry (mass decrease as a function of total light scattered), by particle counters (decrease in numbers), or by chemical means, and the "apparent viable loss" can be adjusted to yield loss of viable cells as if no deposition occurred. Customarily, one plots log of mass or numbers against time, and the slope of the line(s) of best fit is the decay rate.

If the rate of biological decay is found to be less than that of mass loss, then new cells were being formed (6, 8); if the rates are identical, then there was no net death; if the biological decay is greater than mass loss, then death of cells was occurring—a common finding in such experiments.

## MATERIALS AND METHODS

**Aerosol chambers and techniques.** Although rotating-drum chambers at this laboratory and their operation have been described elsewhere (2, 4), a summary is included for the reader unfamiliar with aerobiology methods. Airtight, 1,500-liter, stainless steel drums are rotated (on the axis normal to the ends) at 3 rpm within a temperature-controlled room (±0.1°C); work hoods are maintained at the same temperature. Access (air in-out) is via nonrotating, hollow axles. The supply air is filtered and treated with activated charcoal. Provisions are available for steam injection and subsequent condensation to provide "saturated" air at the drum temperature. Manometers allow the operator to maintain air balance when filling the drum and/or sampling. In experiments to be described, 50 ml of distilled water was injected via a syringe into each drum before each test to assure that the enclosed air would remain essentially saturated at the holding temperature of 30°C. Between tests, drums were "air-washed" for at least 16 h with dry air at 115 liters per min.

**Aerosol generation.** Aerosols were generated in a reflux mode by twin peripheral jets located inside round, glass housings 16 cm in diameter. At 0.703 kg/cm$^2$, they use 9.2 liters of air per min to aerosolize 0.12 g of aqueous liquid, of which 75% is lost by evaporation. Particles larger than 8-μm Stokes diameter do not emerge from the housing, but are impacted and returned to the contained fluid. Aerosols produced from solutions with about 2% solids have mass median diameters of 1.2 to 2.0 μm, with the distribution skewed

toward smaller particles and with some particles ≥4-μm diameter (2). In these tests, the "spray-time" was 5 min.

**Sampling.** Air samples (12.5 liters of air per min) were withdrawn into all-glass impingers (1) containing 20 ml of fluid. Impinger fluid for viability assay was 1% gelatin + 0.01% Dow-Corning Antifoam B in distilled water. Heart infusion agar (Difco) plates were used to assay 0.2-ml portions of each dilution.

**Measurement of volume and numbers of cells.** We used a modified version of the procedure described by Harvey and Marr (5). Samples of the aerosols were obtained in impingers, as described above, and the numbers of cells and their relative size distribution were determined by a Coulter Counter, model B, with a 20-μm orifice.

Collection fluid and electrolyte for counting were 1% (wt/vol) NaCl with 0.5% (vol/vol) Formalin in distilled water. The freshly made fluid was passed through a 0.4-μm-pore-size membrane filter into a 10-liter vessel. By gravity flow, the fluid was passed serially through 0.2- and 0.08-μm Nuclepore filters into 2-liter, plastic, bottom-inlet bottles with air exchange via 0.2-μm-pore-size membrane filters; the bottles were rinsed at least 15 times, or until the "background" at threshold 3 of the counter (aperture current at 1/2; gain maximum) was less than 450 counts per 0.5 ml and less than 20/0.5 ml at threshold 4—a standard adapted for all vessels (impingers, dilution vessels, pipettes, etc.) and fluids in this test. To achieve this count, we chose a room low in acoustical and electrical noise in which to locate the counter. Despite this, electrical noise or a clogged orifice sometimes interfered, and recounts of all samples were done until we were satisfied that acceptable (±8%) reproducibility had been achieved; otherwise, means of three counts that usually agreed within less than ±5% were used. A moving "window" of 2 threshold units was used to obtain the volume-distribution data.

**Culture treatment.** In duplicate, a culture of *Serratia marcescens* (8 UK) was passed every 24 h into 100 ml of minimal mineral salts medium plus glycerol and sodium citrate (11) at 31°C on a rotating shaker by using 100 ml of medium in 500-ml flasks.

For aerosol studies, a flask was removed after 8 h of incubation, the contents were centrifuged for 10 min at 4,000 × *g*, and the pellet was suspended in 20 ml of 0.05 M phosphate buffer (pH 7) plus additional glycerol (1%, vol/vol) and tryptone (1%, wt/vol). Tryptone was used to provide an enrichment material with minimal additional salt content, since it is produced by enzymatic digestion, and tended to minimize osmotic pressure of the solution. The suspension was transferred to the atomizer, and the aerosol was produced as rapidly as possible.

When studies involving the Coulter Counter were to be done, all fluids, including growth medium, were filtered, and glassware was rinsed, as described above.

## RESULTS

Before aerosol studies, we assured ourselves that data from the Coulter Counter were valid. A 24-h culture was inoculated into filtered medium, and samples were removed every hour into the electrolyte; a total count was made with a window of threshold 4 to 20 and compared to a hemocytometer count (American Optical, phase-contrast) of the same sample. Samples were diluted to provide less than 20,000 counts per 0.5-ml sample, because we had found this to be a maximal concentration that showed no coincidence counts. Coincident counts caused not only lowered apparent numbers but also an artifactual increase in modal size. The total counts agreed within ±5% of hemocytometer counts.

The sample was then recounted with a window of 2 threshold units, from threshold 4 to 20, every second unit. The modal threshold value was determined graphically. As the culture grew, the modal value, initially at 6 to 7, changed within 6 h to 11, remained at 11 for an additional 7 h, and then decreased to a value of 6 to 7 again after an incubation period of 20 h. We sometimes observed bimodal distributions (8). Modal values are reported for the larger fraction only.

Because of the difficulties encountered in maintaining the special conditions required to achieve successful analysis of the number and volume of airborne cells, only 2 of 18 sets of tests yielded complete data for all sample intervals. These results are shown in Fig. 1 and 2. The relative mass decay as measured by a light-scatter photometer is shown as a solid line, numbers of viable cells are shown by solid circles, and total cell numbers (Coulter counts) are shown as open circles. The distance A represents the loss of airborne mass within the aerosol chamber during 6 h of aerosol time and is also shown at B to indicate the correction applied to find the true increase in viable cell numbers.

Numbers of viable cells and total cells increased in both instances in a comparable manner. Data shown in Fig. 1 indicate an increase from $7.3 \times 10^5$ to $1.4 \times 10^6$/liter, and those in

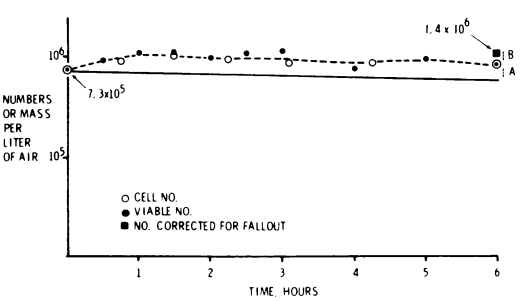


FIG. 1. *Temporal changes in cell numbers, viable cell numbers, and relative mass of aerosols of S. marcescens with glycerol and tryptone held at 30°C and saturated humidity. A is graphical "distance" for correction B.*

Fig. 2 indicate an increase from $7.5 \times 10^5$ to $1.8 \times 10^6$/liter, essentially a twofold increase.

Figure 3 shows data from a control aerosol done with glycerol but without tryptone. The number of viable cells decreased at a rate faster than either the mass of the total cell number.

Figure 4 shows that changes occurred in the modal value of the analog of cell volume for the three aerosols. Cells in the control aerosol decreased steadily in volume, whereas in the test aerosols there was a rapid decrease after 90 min of aerosol time followed by an increase, and then a continued decrease.

## DISCUSSION

In studies of survival of bacteria (2, 6), we had noted instances where numbers of viable, airborne cells had decreased markedly and then apparently increased, sometimes as much as 10-fold. We had never observed instances where the increase exceeded the numbers that would have been present if no cells died, corrected for fallout. Hence, the phenomenon could not be growth. We had suggested that the airborne cells

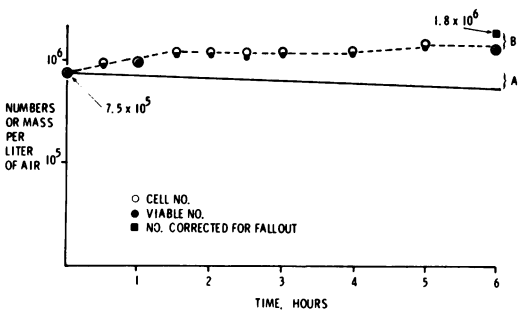


FIG. 2. *Temporal changes in cell numbers, viable cell numbers, and relative mass of aerosols of S. marcescens with glycerol and tryptone held at 30°C and saturated humidity. A is graphical "distance" for correction B.*

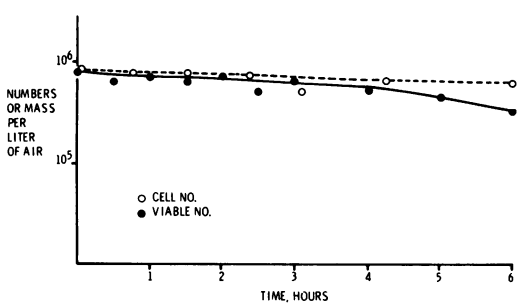


FIG. 3. *Temporal changes in cell numbers and viable cell numbers of aerosols of Serratia marcescens held at 30°C and saturated humidity with no added nutrients.*

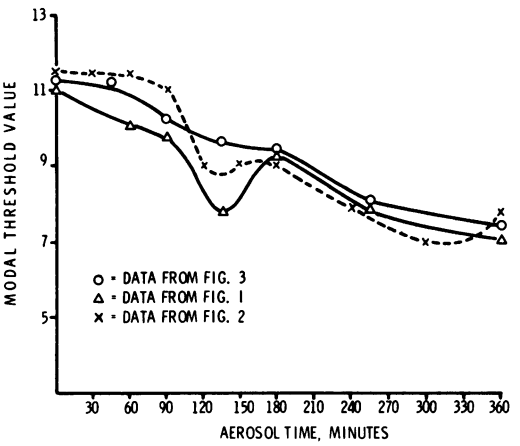


FIG. 4. *Temporal changes in the modal value of the distribution of the analog of cell volume of Serratia marcescens, held as aerosols under conditions noted in the legends Fig. 1, 2, and 3.*

may undergo an interval of "adjustment" wherein they become sensitive to sampling; after adjustment, they again become robust enough to withstand the impact of sampling, and are able to form colonies.

Also, it is a common observation that physiologically "young" cells are more sensitive to almost any stress than "older" cells. Considering these two effects—recuperative capacity and age-related sensitivity—it was not unreasonable to believe that proof of cell division would be difficult. If some new cells were formed, they might die more rapidly than those not growing; if growth occurred during a relatively short interval, the effect could be attributed to recuperation; if growth occurred slowly, then statistical proof would be needed, which would have been convincing to us only if differences were great enough to preclude the need for statistics.

As a result of this reasoning, and after observing increases in "apparent" viability similar to those shown in Fig. 1 and 2, we decided to include the additional study of cell volume, and we continued the tests until we also obtained suitable evidence of an increase in countable cell numbers.

We believe that we have shown conclusively that it is possible for bacteria to undergo at least one division in the airborne state based on the evidence of an almost twofold increase in cell numbers, both countable and viable.

The evidence shows, additionally, that new cells formed during the first 60 to 90 min remained viable for at least 4 additional h. The shift in cell volume, noted as a "saddle" in Fig. 4, is interesting because it indicates that a kind of synchrony (9) existed within the population,

probably caused by the shift-up (10) in the nutritional menstruum, and also illustrates that physiological changes occurred in cells supplied nutrients that did not occur in cells not so treated. That cells increased in volume after an initial, relatively rapid decrease encourages us to believe that continued division might occur if cells were contained in larger particles or were in some way "fed" in the airborne state. We intend to investigate these possibilities.

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