Survival of Bacteria during Aerosolization

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One form of commercial application of microorganisms, including genetically engineered microorganisms is as an aerosol. To study the effect of aerosol-induced stress on bacterial survival, nonrecombinant spontaneous antibiotic-resistant mutants of four organisms, Enterobacter cloacae, Erwinia herbicola, Klebsiella planticola, and Pseudomonas syringae, were sprayed in separate experiments in a greenhouse. Samples were collected over a distance of 15 m from the spray site for enumeration. Spores of Bacillus subtilis were used as tracers to estimate the effects of dilution on changes in population over distance. Viable counts of P. syringae, Enterobacter cloacae, and K. planticola decreased significantly over a distance of 15 m. Erwinia herbicola showed no significant decline in counts over the same distance. The degree of survival of P. syringae during aerosolization was dependent on ambient environmental conditions (i.e., temperature, relative humidity), droplet size of the aerosol, and prior preparative conditions. Survival was greatest at high relative humidities (70 to 80%) and low temperatures (12°C). Survival was reduced when small droplet sizes were used. The process of washing the cells prior to aerosolization also caused a reduction in their survival. Results from these experiments will be useful in developing sound methodologies to optimize enumeration and for predicting the downwind dispersal of airborne microorganisms, including genetically engineered microorganisms.

In recent years, there has been an increased awareness of the environmental applications of genetically engineered microorganisms. The impact of this technology will probably be felt in the field of agricultural biotechnology, most notably, in the areas of microbial control of plant pathogens, weeds, and insects (3, 15), and also in the area of bioremediation. The release of genetically engineered microorganisms into natural environments has raised the issue of proper monitoring and control of these organisms, from a standpoint of both human health and protection of the environment.

Past research on aerosolized bacteria has been done under very controlled conditions in contained laboratory chambers. Data from such studies indicate that aerosolization can cause stress on bacterial populations (2, 4–6, 18). The survival of aerosolized bacteria is influenced by (i) growth conditions prior to aerosolization; (ii) environmental conditions during aerosolization; (iii) methods of aerosolization; and (iv) methods of collection and enumeration (2–8, 12–14). Temperature and relative humidity during aerosolization have been shown to influence the survival of aerosolized bacteria. Escherichia coli K-12 was shown to survive better at low humidities and temperatures than at high humidities when sprayed into an atmosphere of nitrogen (5). Survival, however, was reduced even at low humidities in an atmosphere of oxygen (4). Survival is also dependent on the species of organism being aerosolized.

The only extensive field release study was the release of an ice-nucleation-active strain of Pseudomonas syringae (16). That study indicated that aerosolization may affect the enumeration, survival, and function of bacterial populations and has also raised concerns regarding the survival and efficient detection of genetically engineered microorganisms released into the environment. It is expected that, in many instances, future releases of microorganisms, including genetically engineered microorganisms, will be as aerosols. Therefore, it becomes necessary to study the effects of aerosolization on the survival of microorganisms.

This paper presents the results of an investigation into the effects of aerosolization on the survival of vegetative bacteria and the influence of ambient environmental and culture preparative conditions on their enumeration. Spores of Bacillus subtilis were used as physical tracers to determine the extent of dilution of the aerosolized population, since their survival is not affected by most adverse conditions (12, 17). Four different bacterial genera were tested to evaluate variations in their survival during aerosolization. Results indicate that temperature, relative humidity, droplet size, and washing of cells prior to aerosolization affect the survival of aerosolized bacteria.

MATERIALS AND METHODS

Organisms used. Target organisms used in this study were Enterobacter cloacae EPA 117 (isolated from the gut of a cutworm larva; resistant to 500 µg of nalidixic acid per ml), Erwinia herbicola EPA 81A (supplied by Steve Beer, Cornell University, Ithaca, N.Y.; resistant to 500 µg of nalidixic acid per ml), Klebsiella planticola EPA 658 (isolated from roots of a radish plant; resistant to 100 µg of rifampin per ml), and P. syringae TLP2 (supplied by Steve Lindow, University of California, Berkeley; resistant to 100 µg of rifampin per ml). Spores of B. subtilis ATCC 6537 were used as internal controls to estimate the extent of dilution of the aerosolized population.

Preparation of target cell suspensions. Target organisms were grown at 30°C with shaking at 150 rpm in 600 ml of Luria-Bertani broth (LB broth; Difco Laboratories, Detroit, Mich.) supplemented with the appropriate antibiotic. Antibiotics were used to suppress the growth of indigenous airborne microorganisms and to allow for the selective enumeration of the test microorganism. The organisms were grown to an A600 of 2.0, corresponding to a viable cell density of approximately 10⁶ cells per ml. Cells were washed three times in sterile 10 mM phosphate buffer (per liter of distilled water: 4.0 g of KH₂PO₄, 13.6 g of K₂HPO₄; pH 7.2)
and suspended in 300 ml of sterile distilled water. The washed cell suspension was then added to 2.7 liters of sterile distilled water in a stainless-steel CO2 sprayer (R & D Sprayers, Inc., Opelousas, La.). For experiments with unwashed cells, the bacterial suspensions were centrifuged and suspended directly in sterile distilled water prior to spraying.

Preparation of *B. subtilis* spore suspension. *B. subtilis* cells were grown on plates of nutrient agar (Difco) containing 2.5 ppm (2.5 µg/ml) of MnSO4 for 7 to 10 days at 30°C to induce sporulation. The spores were removed from the plates by suspension in distilled water (10 ml per plate). The spore suspension was then washed three times and suspended in the same volume of sterile distilled water. Prior to spraying, 75 to 100 ml of the washed spore suspension was added to the spray suspension.

Aerosolization procedure. Aerosolization experiments were performed (i) to compare survival of *Enterobacter cloacae*, *Erwinia herbicola*, *K. planticola*, and *P. syringae*; (ii) to study the effects of droplet sizes on survival; and (iii) to study the effects of ambient temperature and relative humidity on survival. All studies on the effects of various environmental and preparative conditions on survival were carried out with aerosolized *P. syringae*. All experiments were conducted in a greenhouse (30 by 9 m). All-class impinger air samplers (AGIs; Ace Glass Inc., Vineland, N.J.) were located at distances of 1, 3, 5, 10, and 15 m from the spray site (Fig. 1). Each AGI contained 20 ml of sterile 10 mM phosphate buffer. The flow rate of air through the critical orifice of the AGIs was 12.5 liters/min. A nonaerosolized control was run upwind of the release site by adding 1 ml of the spray suspension to 20 ml of buffer in an AGI.

The cell suspension was sprayed at a pressure of 35 lb/in² for 2 min. Two different particle sizes were released in separate experiments by using different spray nozzles: (i) Teejet 8004-SS, delivering a median diameter of 450 µm (Spraying Systems Co., Wheaton, Ill.); (ii) Delavan HC 2.70°, delivering a median diameter of 130 µm (Delavan, Inc., Lexington, Tenn.). The AGIs were run during the spray and for a period of 18 min following the spray.

Ambient environmental conditions (temperature, relative humidity, wind speed, and direction) were monitored with various meteorological equipment and stored in a 21XL Datalogger (Campbell Scientific, Logan, Utah). The temperature and relative humidity were adjusted as appropriate for each experiment. The wind speed was approximately 1 m/s and did not vary significantly between experiments. The wind speed was controlled by two large fans at one end of the greenhouse. The sampling setup was adjusted in such a way that the bacterial spray travelled in the direction of the wind, and the AGIs were located directly downstream from the spray source (Fig. 1).

Enumeration of cells. Following aerosolization, aliquots of samples were first plated on LB agar containing the appropriate antibiotics for the enumeration of target organisms. Cycloheximide was added at a concentration of 100 µg/ml to all enumeration media to inhibit fungal growth. To enumerate *B. subtilis* spores, samples were heated for 20 min at 80°C and plated on nutrient agar plates. Plates were incubated at 30°C for 36 to 48 h.

Analysis of data. For each of the experiments described here, data were collected from three separate experiments with six replicates of each. Spores were used as internal controls to determine the extent of dilution of the population. The viable counts of spores were compared with those of target organisms to determine the effects of aerosolization and environmental stresses on the survival of target microorganisms. All data are expressed as target/score ratios. Spore counts were normalized to target cell counts prior to calculating target/spore ratios. Target/spore ratios were calculated by dividing the target cell counts by the normalized spore counts. A change in the target/spore ratio indicates that there is a differential response of the target organism over and above the dilution effect. Thus, a reduction in the survival of the target organism is indicated by a reduction in the target/spore ratio. An analysis of variance was performed on target/spore ratios, using the Statistical Analysis System (SAS Institute, Inc., Cary, N.C.). Ratios were compared between each replicate to determine whether there were any significant variations between replicates. Target/spore ratios were also compared at each of the distances to determine how survival was affected by distance travelled. *P* values of 0.05 or lower were considered significant. All data points in the figures are represented by the mean target/spore ratio of all replicates ± 1 standard error.

Results

The effect of the aerosolization process on the survival of aerosolized *Enterobacter cloacae*, *Erwinia herbicola*, *K. planticola*, and *P. syringae* at 22°C and a relative humidity of 39% is shown in Fig. 2. In this experiment, and all subsequent experiments, the test organism was sprayed out at an initial concentration of ca. 10^5 CFU/ml. There was a reduction of about 4 orders of magnitude in the numbers of cells recovered at the farthest distance (15 m) from the spray site. *Erwinia herbicola* showed no significant reduction in target/spore ratios over the 15-m sampling distance, indicating that there was negligible reduction in the survival of this organism compared with that of the spores. The survival of the other three organisms was significantly lower than that of *Erwinia herbicola* under the same test conditions. The
survival patterns of Enterobacter cloacae, K. planticola, and P. syringae were not significantly different from each other under the conditions tested.

At a distance of 1 m, organisms sprayed by using a large droplet size (median diameter, 450 μm) demonstrated a significantly higher degree of survival than organisms sprayed by using a smaller droplet size (median diameter, 130 μm) at a temperature of 22°C and relative humidity of 39% (Fig. 3). At distances beyond 1 m, there was no significant reduction in the target/spore ratios between the two droplet sizes. Survival at 10 and 15 m was significantly lower when compared with survival at 1 and 3 m for both droplet sizes as indicated by a reduction in the target/spore ratios.

When sprayed as large droplets, there was no significant reduction in the ratios, indicating that survival was higher at an ambient spray temperature of 12°C compared with an ambient spray temperature of 22°C (Fig. 4a). The relative survival at the lower temperature (12°C) was greatly enhanced when a smaller droplet size was used (Fig. 4b).

The experiments described above were done with suspensions of P. syringae which were washed three times in sterile 10 mM phosphate buffer prior to spraying. Unwashed organisms sprayed as large droplets showed no significant decrease in numbers over the entire 15-m sampling distance. However, unwashed organisms sprayed as small droplets showed significantly reduced target/spore ratios at distances of 5, 10, and 15 m (Fig. 5).

**DISCUSSION**

Experiments with four different gram-negative bacteria (Enterobacter cloacae, Erwinia herbicola, K. planticola, and P. syringae) demonstrated that, under similar release conditions (22°C and 39% relative humidity), the different organisms showed different degrees of survival during aerosolization. Erwinia herbicola showed the highest level of survival at ambient temperatures of 22°C (Fig. 2). There were no significant differences in the survival of the other three organisms.

Under our test conditions, the detrimental effects of aerosolization on the survival of washed P. syringae were...
independent of the size of the droplets carrying the bacteria at a higher ambient temperature (22°C; Fig. 4). At an ambient temperature of 22°C and a relative humidity of 39%, the rate of evaporation of droplets is very rapid, even for large droplets (10 to 30 sec; W. Yates, personal communication). Upon evaporation, bacterium-carrying droplets reach a critical final size beyond which there is no further reduction in size. This critical size is dependent on ambient environmental conditions (B. Lighthart, B. T. Shaffer, B. Marthi, and L. Ganio, unpublished data). The absence of any significant differences in the survival of P. syringae between the two droplet sizes at 22°C is probably indicative of the fact that the final particle size in both cases is similar.

The rate of evaporation, which is a function of ambient temperature and relative humidity, is reduced at the lower temperature (12°C), since the relative humidity is high (77% under the conditions tested). A relative humidity in the range of 70 to 80% has been shown to have a protective effect on aerosolized bacteria (8, 11). The protective effect seen in Fig. 4 may, therefore, be a combined effect of lower temperature and higher relative humidity. When experiments were conducted with small droplet sizes at an ambient temperature of 12°C, results showed a higher relative target/spore ratio at distances of 10 and 15 m than at distances of 3 and 5 m. It is thought that this may be due to clumping together of B. subtilis spores, used as controls in all experiments, due to the effects of the increased humidity. This may underestimate the number of spores counted and also the relative ratio of target organisms to spores. A high relative humidity may also cause clumping together of vegetative cells and possibly increase survival.

Preparative procedures prior to aerosolization had a profound effect on the survival of P. syringae (Fig. 5). These experiments were done under the same conditions of temperature and relative humidity (22°C and 39%) as the experiments described in the legend to Fig. 2. Unwashed cells sprayed in large droplets did not show any significant decrease in survival over a distance of 15 m (Fig. 5). Washed cells aerosolized under the same conditions showed a significant reduction in survival (Fig. 3). Unwashed cells sprayed as small droplets, however, showed significantly reduced survival. The presence of organic matter from the growth medium (LB broth) increases the vapor pressure of droplets.

This might reduce evaporation and confer protection to the aerosolized bacteria. However, this protective effect may be masked by the more rapid evaporation of smaller droplets, thus reflecting the greater die-off seen in Fig. 5. Due to the short (20-min) collection time in the AGIs and the absence of any nutrient medium (cells are collected in phosphate buffer), the possibility that cells may be multiplying is remote.

Additional experiments were done to determine whether the washing procedure caused any reduction in the viable counts of these bacteria. Viable counts of an unwashed suspension of P. syringae were compared with viable counts of the organism washed three times with 10 mM phosphate buffer (pH 7.2). Results showed that there was no significant difference in the viable counts of unwashed and washed cells (data not shown). This indicates that the washing procedure itself did not affect viable counts of these bacteria. Thus, any differences seen in the survival of washed and unwashed bacteria after aerosolization were due to the stress of the aerosolization process itself.

In summary, of the four genera used in this study, Erwinia herbicola showed the highest degree of survival during aerosolization over distances of 15 m. There was no significant difference in the survival of Enterobacter cloacae, K. planticola, and P. syringae.

The survival of aerosolized P. syringae was found to be dependent on a number of factors, including droplet size, ambient temperature, and relative humidity, and culture preparative procedures.

This study is useful for the development of monitoring protocols for detection of released microorganisms, including genetically engineered microorganisms. Such protocols would include the use of media and conditions that would better allow stressed microorganisms to be detected. Results from such studies can also be used to model the downwind dispersal of aerosolized bacteria by predicting the effects of various environmental and preparative conditions on the survival of aerosolized bacteria.

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