Survival of Airborne Bacteria in a High Urban Concentration of Carbon Monoxide

BRUCE LITHTHART

Institute for Freshwater Studies and Department of Biology, Western Washington State College, Bellingham, Washington 98225

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Vegetative cells of Serratia marcescens 8UK, Sarcina lutea, and spores of Bacillus subtilis var. niger were held in aerosols, with and without an urban concentration of CO (85 μleters per liter or ppm), for up to 6 hr at 15 C and a relative humidity (RH) of approximately 0, 25, 50, 75, and 95%. It was found that CO enhanced the death rate of S. marcescens 8UK at least four- to sevenfold at low RH (ca. 1 to 25%), but protected the cells at high RH (ca. 90%). Death rates of S. lutea, with or without added CO, were comparatively low over the entire RH range. However, in the first hour, airborne S. lutea held in CO-containing air were more stable than those in air without added CO (i.e., CO protection). A marked increase in the death rate (up to 70-fold) occurred in the subsequent 5 hr within the RH range of approximately 0 to 75%. Statistical analysis indicated that aerosol decay rates of B. subtilis var. niger spores decreased significantly, when held in a CO-containing as compared to a non-CO-containing atmosphere, in the 0 to 85% RH range. Thus, the data presented indicate that CO in the urban environment may have a protective or lethal effect on airborne bacteria, dependent upon at least the microbial species, aerosol age, and relative humidity. A mechanism for CO death enhancement and protection of airborne S. marcescens 8UK is suggested to involve CO uncoupling of an energy-requiring death mechanism and an energy-requiring maintenance mechanism at high and low RH, respectively.

CO is disseminated into the urban atmosphere from various industrial sources, primarily internal combustion engines (28), at a rate estimated to be 200 million metric tons of CO per year (26). Local concentrations of CO beyond the planetary background level of approximately 0.3 to 0.90 parts per million (34) accumulate in areas of high urban vehicular traffic such as Los Angeles and New York, where levels of 72 ppm (1) and 20 ppm (4) are found, respectively. Removal of CO from the atmosphere may not be the result of atmospheric oxidation alone (10), as the soil microflora is thought to be a natural sink where many atmospheric pollutants are decomposed (22).

Bacteria enter and are dispersed into the atmosphere from various sources such as soil, skin flakes, sneezes, talk, sewage treatment plants, wind-blown ocean spray, and various urban activities (e.g., 19). While in the airborne state, their survival with time is a function of, among other factors, temperature (15), relative humidity (20, 31), and atmospheric chemical composition (7, 8, 12, 21, 25). Pollutant gases common in the urban atmosphere have been used for various reasons to kill bacteria, e.g., formaldehyde (33), nitrogen dioxide (23, 33), PAN (27), acrolein, ozone (16), and sulfur dioxide (29, 33). Lighthart et al. (25) studied the survival of Serratia marcescens (ATC 274) at a mid-to-high relative humidity (RH) range in urban concentrations of sulfur dioxide and concluded that the death rate of these bacteria increases as the gas concentration increases and the RH decreases, and decreases with cloud age.

The death mechanism(s) of airborne vegetative gram-negative bacteria (such as S. marcescens 8UK and Escherichia coli K-12) is thought to be divisible into an RH-dependent mechanism in the high RH range and an oxygen-dependent mechanism in the low RH range (2). The former mechanism is thought to

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involve either a nonoxidative process in which ribonucleic acid–protein complex dissociates and is rendered active for subsequent ribonuclease degradation, or protein synthesis and energy production are inactivated (8, 9). Webb (32) hypothesizes that a “dark” repair mechanism similar to that in ultraviolet-induced damage might be operating at high RH. At low RH, the death mechanism is apparently associated with oxidative cellular processes, as indicated by direct linear dependence of cellular death rates on oxygen concentration (21, 35) and high survival in relatively inert gases (7–9). The low RH death mechanism appears to involve oxygen-induced effects, similar to those affecting freeze-dried E. coli B, which involve the formation of free radicals in the presence of oxygen (11). Marked reduction in the death rate of E. coli strains and S. marcescens 8UK suspended at low RH in a nitrogen atmosphere (7.9) further support this notion. Webb (32) presents further evidence of oxygen-induced effects, and suggests that some easily oxidized cellular constituents in the cytochrome system might be the lethal site. Benbough (3) presents evidence for the hypothesis that death of dried E. coli B in an oxygen-containing environment is induced through oxygen-mediated free radical formation in the pyridine nucleotide-flavoprotein cytochrome oxidase system. He suggests that a flavoprotein may be the specific radical formation site.

CO affects cellular metabolism by reacting with the cytochromes, forming a photosensitive carboxyl compound in some organisms (6, 14, 24, 30). Cairns and Denhardt (5) have shown that in vivo bacterial chromosome replication depends, at all times, upon some CO-sensitive reaction which is not required for the first steps of certain phases of replication.

This communication will present the results of laboratory work to elucidate the effect of a high urban concentration of CO on the survival of the vegetative bacteria, S. marcescens 8UK, Sarcina lutea, and the spores of Bacillus subtilis var. niger at a given temperature and several relative humidities.

MATERIALS AND METHODS

Aerosols of a mixture of S. marcescens 8UK, S. lutea, and spores of B. subtilis var. niger were stored in a 709-liter stainless steel drum, rotating at 3 rev/min (17, 18). The drum was light tight, and was maintained at 15 ± 1 C in a constant temperature room.

The cell spray suspension was prepared by mixing second passage cultures of S. marcescens 8UK grown in 250-ml flasks containing 50 ml of Trypticase soy broth (BBL) for 24 hr, and S. lutea grown in 500-ml flasks containing 100 ml of Trypticase soy broth for 48 hr. Both cultures were incubated at 15 C on a reciprocating (200 rev/min) shaker. Prior to mixing, bacteria were washed and suspended in sterile distilled water. The cell suspension of S. lutea was homogenized in a Waring blender for 1 min to break up cell packets before mixing. Sterile distilled water suspensions of B. subtilis var. niger (Ft. Detrick lot no. 10-117) were added to the S. marcescens 8UK and S. lutea suspension to give final concentrations of 10⁹ to 10¹⁴ colony-forming units (CFU) per ml of each species. The cell suspension was sprayed into the drum by a modified Wells reflex atomizer (12), operated at 15 psi for 1 min. Atomization was commenced 4 min prior to cell introduction into the drum.

Aerosol samples were collected with AGL-30 impingers containing 20 ml of sterile distilled water, and operated at 12.5 liters per min for 30 or 60 sec. Viable cell counts were determined on 10-fold dilutions of the impinger fluids. The bacteria were spread on the surface of Trypticase soy agar plates containing 0.15 µg of Brilliant Green per ml for assay of S. marcescens 8UK, and on Trypticase soy agar containing 0.15% phenethyl alcohol for assay of S. lutea. Assay of B. subtilis var. niger spores was made on impinger fluid heated to 70 C for 5 min and then spread on Trypticase soy agar plates. Duplicate sets of all plates were incubated at 35 C for 24 hr for S. lutea. All inoculation procedures were performed in a laminar sterile air-flow hood.

The drum was preconditioned to the desired relative humidity by passing activated charcoal and Millipore-filtered (0.45 µm porosity) air through the drum for up to 10 hr prior to introduction of the test gas or bacterial aerosol. Fifteen minutes prior to aerosol production, research grade CO (Matheson Gas Products, East Rutherford, N.J.) was injected with a glass syringe into the drum, to give a final drum concentration of 85 µlites of CO/liter (85 ppm). Immediate internal mixing of the aerosol in the drum was facilitated by an on 45-off 15-sec cycle 4 min before and after filling the drum.

Relative humidity and temperature inside the drum were monitored with a hygrometer indicator model 15-3001 (Hydrometrics, Inc., Silver Spring, Md.).

Calculations. The percent survival of the test organisms was calculated as the quotient of the total aerosol decay and physical decay times 100: Per cent Survival = (Total decay/Physical decay) × 100 = [N(t)/N(0)]/(N(0)/N(t₀)) × 100, where N(t₀) = number of CFU of the test organism at time T; N(t) = number of CFU of the test organism in the initial sample; and N₀ and N₀ + number of tracer spore CFU initially and at time T. The death rate (-log per cent hr⁻¹) was calculated as the negative slope of a line fitted by the method of least squares to the experimental points, giving the logarithm of the per cent survival after the appropriate time intervals. Decay rates for 0 to 1- and 1 to 6-hr aerosol incubations are designated K₁ and K₂, respectively. All calculations were made by using the BMD series computer programs (13).
RESULTS

The death rates of air-borne S. marcescens 8UK, without added CO, increased inversely as the RH decreased from a range of approximately 88 to 95% to 1.2 to 3.0% (Table 1). During the first hour, a sixfold differential in rates of death was observed between the highest and lowest humidity ranges. In the following 5 hr, there was a similar 6.5-fold increase in the death rate occurring in at least the 50 to 75% RH range.

Upon addition of CO to aerosols held at a high RH range, the decay rates (K₁) of S. marcescens 8UK increased within the first hour from a low value, which was less than observed with no added CO, to a sixfold increase in the RH range of 75 to 90% (Table 1). At the lower RH ranges (1-50%), there was a further 19-fold increase for an overall increase of 114 times in the death rates from high to low RH. There is a strong indication that the increase in the vicinity of 25% RH is not a maximum value. Due to the rapid die-off of S. marcescens 8UK in CO at low RH, a viable count was not attainable after the initial 4-min sampling; therefore, decay rates were calculated for the minimum CFU detection level.

For cells of S. marcescens 8UK held for 1 to 6 hr in a CO-containing atmosphere, the death rate (K₂) increased again, but only 1.7-fold in the 75 to 90% RH ranges. This is an approximately threefold lower death rate than observed during the first hour in the aerosol. Due to the rapid loss of viability of S. marcescens 8UK when incubated in 85 ppm of CO at less than 75% RH, no viable cells were recovered from samples collected beyond 1 hr.

To illustrate the effect on the survival of airborne S. marcescens 8UK, a ratio of the death rates, with and without added CO (calculable from Table 1), was determined. A ratio value of one indicates no effect of added CO, greater than one indicates a lethal effect, and less than one indicates a protecting effect of CO. During the first hour in the aerosol, the ratio for S. marcescens 8UK increased from 1.26 at 75% RH to >7.71 in the 1 to 25% range, showing a marked lethal effect of CO at low RH. The decrease in the ratio to much less than one at greater than 88% RH indicates a protecting effect at high RH. During the subsequent 5 hr, the ratios showed that the lethal action of CO increased further at high humidities, but not enough to negate the protective effect at the highest RH levels tested.

During the first hour after atomization, the death rates (K₁) for S. lutea without added CO were up to 10 times lower over the RH range of 23.4 to 96% when compared to those found for S. marcescens 8UK (Tables 1 and 2). At the lowest RH range, 1.2 to 3.4% RH, the death rate for S. lutea increased significantly. In the subsequent 5 hr, the rates increased significantly at both the extreme high and low RH ranges, and decreased to a minimum at approximately 50% RH.

Upon addition of an S. lutea aerosol to an atmosphere containing 85 μl/lt of CO per liter, the first hour death rates (K₁), with an exception at the lowest RH range, decreased to less than the control atmosphere with no added CO, indicating a protecting effect of CO. In the subsequent 5 hr, the death rates (K₂) increased dramatically, up to 5.7-fold, in the 25 to 75% RH ranges.

The effect of added CO, as indicated by the death rate ratio (calculable from Table 2) of S. lutea, shows that CO decreases the death rate in the first hour (K₁), except near 0% RH, and produces an increase in cell death rate about 25% RH in the following 5 hr (K₂). There appears to be a peak lethal effect near 25% RH.

### Table 1. Mean logarithmic death rates of aerosolized Serratia marcescens 8UK while incubated for <1 (K₁) and ≥1 (K₂) hr at the indicated RH ranges, with and without 85 μl/lt of CO at 15 C

| RH range during experiments (%) | Without CO | With CO |
|--------------------------------|------------|---------|
|                                | No. of tests | K₁ | K₂ |
| 1.2-3.4                        | 3           | 2.690 | 10.828 |
| 23.4-26.5                      | 3           | 2.916 | >225  |
| 45.0-51.5                      | 3           | 0.848 | 0.368 | 1.520 |
| 73.0-75.5                      | 2           | 0.450 | 0.057 | 0.570 | 0.103 |
| 88.0-96.0                      | 2           | 0.404 | 0.090 | 0.090 | 0.061 |

*Minimum detection level.

### Table 2. Mean logarithmic death rates of aerosolized Sarcina lutea while incubated for <1 (K₁) and ≥1 (K₂) hr at the RH ranges, with and without 85 μl/lt of CO at 15 C

| RH range during experiments (%) | Without CO | With CO |
|--------------------------------|------------|---------|
|                                | No. of tests | K₁ | K₂ |
| 1.2-3.4                        | 4           | 0.165 | 0.346 | 5 | 0.295 | 0.314 |
| 23.4-26.5                      | 3           | 0.039 | 0.018 | 2 | -0.023 | 1.326 |
| 45.0-51.5                      | 3           | 0.020 | 0.001 | 2 | -0.035 | 0.060 |
| 73.0-75.5                      | 2           | 0.026 | 0.025 | 3 | -0.291 | 0.050 |
| 88.0-96.0                      | 2           | 0.021 | 0.018 | 2 | -0.022 | -0.023 |
where the cell decay rate was approximately 70 times higher than at the same RH without CO. F tests for each decay rate, with and without added CO, were significantly different at the 99% confidence level.

The decay rate constants for *B. subtilis* var. *niger* spores were determined at relative humidities greater than 85% RH and equal to or less than 85% RH, with and without added CO (Table 3). The decay rates for the spores held at the high range as opposed to low, with or without added CO, were significantly less, as indicated by F-tests carried out on the standard error of the regression coefficients. Moreover, the decay rates at the high RH range, with and without added CO, showed no significant differences, whereas at low RH there was a highly significant decrease in the decay rate with added CO.

The magnitude of the loss of spore viability in tests with CO was so small compared to vegetative cell death rates that it was not taken into account in the death rate calculation of *S. marcescens* 8UK or *S. lutea*.

**DISCUSSION**

The results of this investigation show that airborne *S. marcescens* 8UK, *S. lutea*, and spores of *B. subtilis* var. *niger* are affected by the environmental parameters, relative humidity, and CO. For example, *S. marcescens* 8UK was relatively stable at relative humidities above 75%; their rate of death increased rapidly between 50 and 25% RH, and remained high below 25%. At an aerosol age of 1 to 6 hr, those bacteria maintained at greater than 50% RH, for some unknown reason(s), lose viability up to 7.5 times slower than during their first hour in an aerosol at similar RH. Studies of aged aerosols of *S. marcescens* in atmospheres containing 85 mliters per liter of CO indicated that cells were protected from loss of viability at approximately 88% RH and above, whereas below 75% RH the death rate increased from approximately 4 to greater than 20 times that observed when CO was not present.

Although the death rates without CO for *S. lutea* were markedly less than for *S. marcescens* 8UK, there was a distinguishable increase in the death rate for *S. lutea* at very low RH during the first hour which, when CO was added, increased even further. At high RH CO appeared to protect the cells, particularly at 75% RH. The positive regression slopes or death rates calculated for *S. lutea* with added CO may be due to several reasons: (i) *S. lutea* cells "killed" prior to withdrawal of the first sample subsequently repaired themselves; (ii) there was incomplete breaking of the sarcina during blending, and the CO stimulated package break-up, yielding more airborne cells with time; or (iii) the tracer-spore death rate in CO at high RH significantly affected the calculation of the biological decay rates of *S. lutea*.

Statistical analyses indicate that the *B. subtilis* var. *niger* viable spore loss rate is greater below 85% RH than above, with and without added CO, and spore loss at low RH is significantly less in the presence of 85 ppm of CO.

The data presented in this study support the previous views on the death mechanisms in vegetative cells. Upon addition of an aerosol of bacterial cells possessing cytochrome enzymes into a dark atmosphere containing a high urban concentration of CO, it would be expected that those processes requiring energy derived in the electron transport system would be markedly affected by inhibition due to the formation of a CO-carbonyl compound. The effect of CO addition at high RH in both types of vegetative cells could be to inhibit a small energy-requiring death mechanism, or to "re-route" conserved energy to a repair mechanism, thus protecting the cell from a lethal action, whereas at low RH, energy is necessary to remain viable (e.g., energy for repair and maintenance), and that CO-interrupted energy flow results in cell death.

The difference between cell types in CO sensitivity may be due to the energy-deriving mechanisms in these bacteria; e.g., both *S. marcescens* 8UK and *S. lutea* have cytochrome systems that will be inhibited by CO, but only *S. marcescens* 8UK can carry on further energy generation via the glycolytic pathway. Continuous operation of the glycolytic pathway in

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**Table 3. Tabulation of aerosol decay rates and F-test comparisons of Bacillus subtilis var. niger spores at the indicated RH ranges and CO concentrations at 15 C**

| Sample no. | Sample size | RH range (%) during experiment | CO concn (liter per liter) | Regression coefficient (i.e., β)* |
|-----------|-------------|--------------------------------|---------------------------|----------------------------------|
| 1         | 73          | 0–85                           | 0                         | 0.019*                           |
| 2         | 27          | 85–100                         | 0                         | −0.001                           |
| 3         | 68          | 0–85                           | 85                        | 0.0003                           |
| 4         | 14          | 85–100                         | 85                        | −0.012                           |

*F* samples 1, 2 = 5.45; *F* samples 2, 4 = 4.26; *F* samples 1, 3 = 1.85 (preceding numbers indicate a significant difference at the 99% confidence level); *F* samples 2, 4 = 1.45 (not significant).

* Aerosol loss rate in −log per cent per hour.
the presence of oxygen may result in deleterious metabolic by-products in the cell. It appears that CO may penetrate and act to protect spores of *B. subtilis var. niger* at low RH by unknown mechanisms.

The implications of this investigation, with reference to the effects of CO in urban concentrations on the survival of ambient airborne bacteria, indicate that, depending upon relative humidity, some vegetative cells that have similar airborne properties to bacterial pathogens may be rapidly killed or protected from drying, whereas at least one spore type was either not affected or protected by CO.

The results of this investigation indicate that more research of this nature must be carried on before an adequate understanding of the effects of airborne air pollutants on the survival of airborne microorganisms is obtained. It is anticipated that the effects, including synergistic effects, of various kinds of microorganisms and the quality and quantity of pollutant gases, temperature, and light quality as well as quantity during incubation, can and must be evaluated. Data derived from these investigations may then be used, in conjunction with predictive atmospheric dispersion models, to give estimates of viable cell concentrations downwind from point sources such as sewage treatment plants, hospitals, rendering plants, phytotrophic sites, and humans, among others (Lighthart and Frisch, *in press*).

It is anticipated that field work will be encouraged to confirm some of the laboratory findings. An ideal example for a field study may be in the vicinity of activated sludge or trickling filter treatment plants downwind of a large pollution-producing urban area. The investigation might involve the multivariate analysis of estimates of airborne cells, air pollutant content, and relative humidity occurring about treatment plants on quiet nights.

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