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Relative Humidity and the Killing of Bacteria

The Survival of Damp *Serratia marcescens* in Air

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Abstract

Bateman, J. B. (U. S. Army Chemical Corps, Frederick, Md.), Patricia A. McCaffrey, R. J. O'Connor, and G. W. Monk. Relative humidity and the killing of bacteria. The survival of damp *Serratia marcescens* in air. Appl. Microbiol. 9:567–571. 1961.—The viability of washed moist cells of *Serratia marcescens* after storage has been measured in relation to variations in the prior treatment of the cells and in conditions of storage. The factors considered were: (i) water content during storage; (ii) method of arriving at water content (partial drying in vacuum or freeze-drying and addition of water); (iii) presence or absence of air during storage.

Increasingly rapid decay occurs as the water content at which the cells are stored is diminished from above 90% to 20 or 30% ("critical" water content). It occurs in presence or absence of air and it occurs whether the final water content is approached by removal of water from wet cells or by addition of water to freeze-dried cells.

The rate of decay during storage at 20 to 30% water is somewhat diminished by the presence of air ("protective" effect of air).

As the water content is further reduced to less than 10%, the stability of cells stored in a vacuum approaches that of wet cells. In presence of air the reverse is true: the stability decreases until at less than 1% water, the decay rate is about as great as at the "critical" water content ("toxic" effect of air).

Particularly rapid decay of *S. marcescens* at the "critical" water content has escaped attention in aerosol studies because accurate control of relative humidity (RH) in this region, RH 94 to 99%, is virtually impossible in such studies. On the other hand, values of decay rates referred to measured water contents are quite unreliable in the 20 to 80% RH zone because the corresponding variation of water content is too small to measure reliably. Thus data of the kind reported in this paper cannot be directly compared to the published results of studies of air-borne bacteria, although they are relevant to the practical question of air-borne infection in humid atmospheres.

When washed and freeze-dried *Serratia marcescens* is stored in water vapor in vacuum, there is a zone of relative humidity (RH), around 90 to 95%, within which the bacteria die quite rapidly (Monk and McCaffrey 1957). This paper describes similar experiments with freeze-dried cells moistened and exposed to air, and with damp cells stored in air without prior freeze-drying.

Dunklin and Puck (1948) found that pneumococci sprayed from broth suspension rapidly lost viability at ambient 50% RH, whereas at greater or lower humidities they were relatively stable. Similar, although less pronounced, effects were found with *Staphylococcus albus* and group C *Streptococcus hemolyticus*. This striking humidity dependence was eliminated when cells were sprayed from salt-free suspensions, and was attributed to the action of concentrated salt solution upon cells at a "critical" degree of dehydration; cells not containing the postulated "critical" amount were presumed less sensitive to toxic agents.

A qualitatively similar zone of rapid decay was discovered by different methods by Monk et al. (1956). These investigators freeze-dried thin layers of *S. marcescens* suspended in Naylor-Smith medium (Naylor and Smith, 1946) and added water either by injection or by equilibration at constant RH. The moistened cells were stored in vacuum and resuspended for assay of viability. The maximal attenuation occurred when the water content was about 50%, corresponding to an RH greater than 94%, in contrast to the "critical" 50% RH value found by Dunklin and Puck for other microorganisms sprayed into air as a suspension in broth. With washed *S. marcescens* (Monk and McCaffrey, 1957), the "critical" zone was still evident, although the maximal death rate at 94% RH was about one-fifth of that in the presence of Naylor-Smith medium. To account for this behavior, Monk, McCaffrey, and Davis (1957) postulated an unidentified intracellular substance, toxic in high concentrations up to the limit of its solubility in water, the equilibrium RH of the saturated solution being 94%. Thus the Dunklin-Puck postulate of a substance of concentration-dependent toxicity and limited solubility was retained in modified form, while their second postulate

of a "critical" or especially sensitive degree of cell dehydration was tacitly discarded.

The poor survival of washed *S. marcescens* under the conditions of Monk and McCaffrey's experiments, whether attributable to an intrinsic toxic factor or to an undefined "deleterious" physical-chemical condition of the cells, thus presents a contrast to the behavior of other types of microorganisms in Dunklin and Puck's experiment with washed cells. The present experiments were done in an attempt to determine whether in presence of air washed *S. marcescens* would show the flat response to RH change characteristic of the cell types used by Dunklin and Puck.

MATERIALS AND METHODS

Cells of *S. marcescens*, strain 8UK, grown for 18 to 24 hr in Bacto-tryptose broth[1] (26 g per liter) were used to inoculate Roux bottles containing tryptose agar (26 g per liter Bacto-tryptose broth + 20 g per liter agar). After 18 to 24 hr of growth at 31 C, the cells were harvested in distilled water, centrifuged, and resuspended in distilled water. The final viable cell concentration was about 2×10^{11} per ml.

In experiments involving addition of water to freeze-dried cells and storage in air or in vacuum, 0.5-ml portions of washed stock suspensions were placed in one leg of each of eight inverted U-tubes of the type shown in Fig. 1 of Monk and McCaffrey (1957). They were shell frozen and dried for 1 hr. Air was admitted. The tubes containing the ice from the samples were removed and replaced by fresh tubes containing measured amounts (0 to 100 µl) of ice. The system was again evacuated and the sample tubes cooled to −80 C. The ice sublimed rapidly to the sample tubes, which were then warmed to room temperature. After 15 min air was admitted to some sample tubes, whereas others remained under vacuum. One sample tube was detached immediately and the cells diluted with distilled water and plated; the remainder was left at room temperature for the desired storage period. One tube in each experiment was always reserved for determination of water content by drying to constant weight.

In experiments on cells partially dried in air, 3 ml of a stock suspension of washed cells were placed in a round-bottomed flask, immersed in a water bath at 25 C, attached to a Rinco[2] rotating laboratory evaporator. The evaporator was connected through a cold trap (Dry Ice and alcohol) to a three-way stopcock and a mechanical vacuum pump. After evaporation had continued for the desired length of time (2 to 30 min, to obtain water contents between 10 and 90%), air was admitted. The flask was stoppered and weighed, and left at room temperature (24 to 26 C) until opened for assay of viability.

[1] Difco Laboratories, Inc., Detroit, Mich.
[2] Rinco Inst. Co., Greenville, Ill.

All viable counts were the result of triplicate plating on nutrient agar following tenfold serial dilutions.

RESULTS

Viability of moist freeze-dried S. marcescens after storage for 1.5 hr in air or in vacuum. The results were analyzed in three stages: (i) The 15-min equilibration in vacuum resulted in substantial losses of viable count which varied with the final water content (Fig. 1). For water contents between 0.2 and 0.5 g per g wet cells, the viable counts were between 0.1 and 0.01 of the control; for water contents less than 0.2 and greater than 0.5, the relative counts were between 0.1 and 1.0. This statement is valid for all 23 experiments performed. (ii) Taking the 15-min count in each experiment as the initial value N_0, the relative viable counts after 1.5 hr of storage in air decreased with decreasing water content as shown in Fig. 2.[3] The values in the 20 to 30% zone of water contents correspond roughly to first order decay constants of 1 to 3 hr^{-1} compared to 4 to 5 hr^{-1} found by Monk and McCaffrey (1957) for washed cells in vacuum. (iii) In Fig. 3 the 1.5-hr counts in air are expressed as fractions of those in vacuum. This method of presentation shows an effect of air in stabilizing the cells within the "critical" zone and toxicity for very dry cells.

Rates of attenuation of dry and moist freeze-dried S. marcescens during storage in air. The data were separated into two groups, one referring to freeze-dried cells

[3] Some of the data included in Fig. 2 have been presented briefly in different form by Mallett et al. (1959).
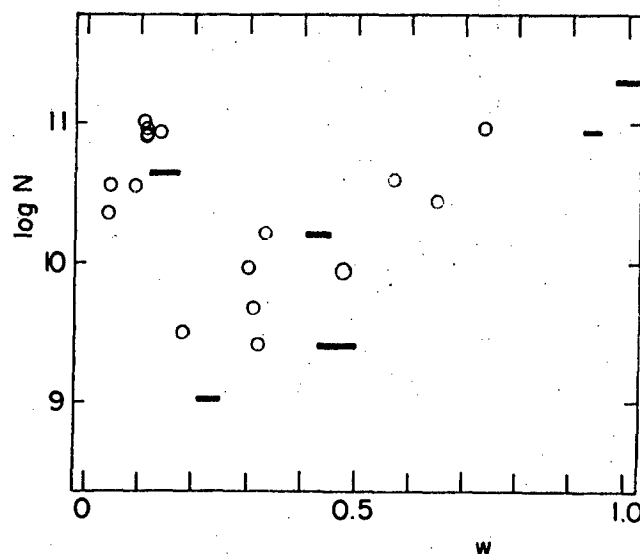

FIG. 1. *Loss of viability of freeze-dried Serratia marcescens following addition of water and storage in vacuo. Abscissa: water content in grams water per g of moist sample. Ordinate: logarithm of viable count after 15-min equilibration period. Black horizontal bars represent the results of those experiments in which duplicate measurements of sample water content gave appreciably different results.*

(4 to 13% H₂O) and the other to freeze-dried cells to which water had been added by sublimation (20 to 30%). The results (Fig. 4) are compared with the corresponding decay lines calculated from data of Monk and McCaffrey (1957) for cells in vacuum.[4] The average decay rates in air are not greatly different for the two ranges of water content, in contrast to those determined in vacuum by Monk and McCaffrey. The toxic effect of air at the lower water contents is thus confirmed. For the partially rehydrated cells of water content 20 to 30%, there is a somewhat lower decay rate than that found by Monk and McCaffrey, suggesting a certain degree of protection in presence of air, in agreement with the results already shown in Fig. 3 by direct comparison of viable counts in air and in vacuum.

[4] The comparison of the present data with those of Monk and McCaffrey is justified by the fact that our data and theirs were obtained in the same apparatus, by the same laboratory personnel using the same procedures, and with cells grown from the same seed stock. There is abundant evidence that no radical change took place in the properties of the stock culture between the two series of experiments.
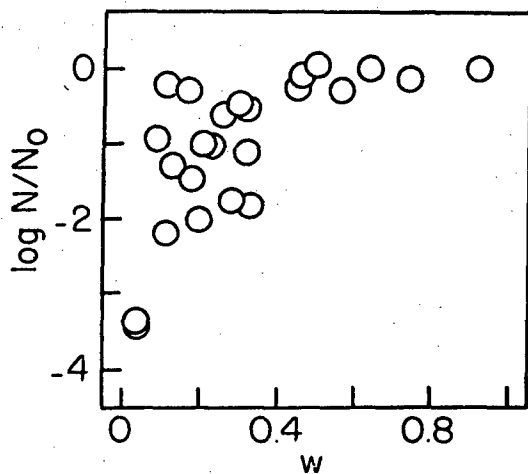
Attenuation of S. marcescens exposed to air after partial drying in vacuum. Two sets of experiments were done, with storage periods close to 1 and 5 hr, respectively. The fractional recoveries are plotted against final water content in Fig. 5. The data for 5 hr of storage leave no doubt as to the relatively rapid decay at about 30% water. This is less evident for 1 hr of storage, partly because the loss factors are less than 10 and also because a substantial part of the loss measured after 1 hr of storage may have occurred during the period of evaporation. A rough idea of the maximal decay rate can be obtained from the difference in recoveries between 1 and 5 hr at 27% water; this gives a first order constant k of about 1.15 hr⁻¹. Thus, there appears to be a definite water content zone of relatively rapid decay, even in cells which have never been freeze-dried,

FIG. 4. *Death of freeze-dried washed Serratia marcescens in air. Abscissa: time of storage in air. Ordinate: negative logarithm of proportion of original cells surviving, N/N_0, where N_0 is initial viable count. The circles on the left are for freeze-dried cells without added water, water contents 4 to 13%. Circles on the right are for freeze-dried cells plus sublimed water, water contents 20 to 30%. Lines represent average decay rates observed by Monk and McCaffrey (1957) for freeze-dried cells stored in vacuo.*

FIG. 2. *Loss of viability of freeze-dried Serratia marcescens following addition of water and storage for 1.5 hr in air. Abscissa: water content in grams water per g moist sample. Ordinate: logarithm of fractional viable count N/N_0 where N_0 is viable count after the 15-min equilibration period.*
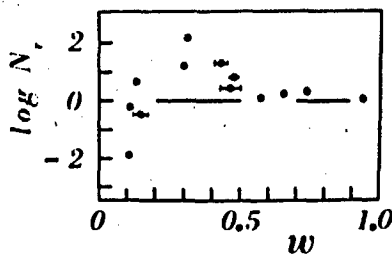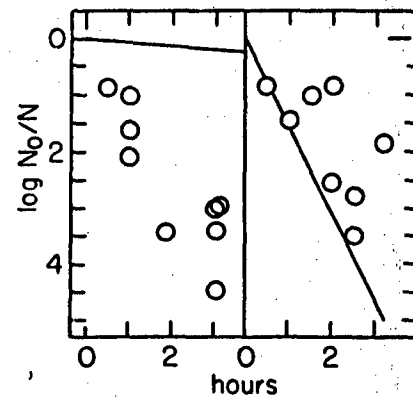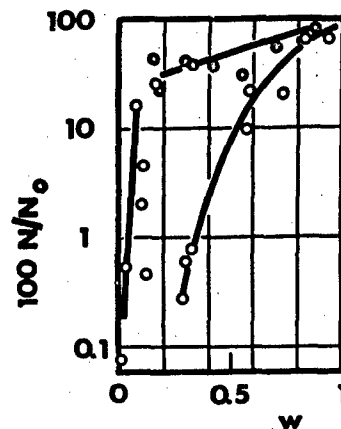

FIG. 3. *Loss of viability of freeze-dried Serratia marcescens following addition of water and storage for 1.5 hr. Abscissa: water content in grams water per g moist sample. Ordinate: logarithm of relative viable count N_r, the ratio of the viable count of cells stored in air to that of cells stored in vacuo.*

FIG. 5. *Survival of Serratia marcescens partly dried by evacuation at room temperature and stored in air. Abscissa: water content, grams water per g moist cells. Ordinate: fractional viable count, logarithmic scale. Double circles represent values for cells stored in air for about 1 hr. Single circles are for cells stored in air for about 5 hr.*

during storage in air; k is, however, lower than the value found by Monk and McCaffrey (1957) (~ 4 hr^{-1}) for freeze-dried cells in vacuum.

At very low water contents, air again appears to be toxic. The 5-hr recoveries diminish sharply as the water content decreases from 7 to 0.2%. There is a poorly defined intermediate region, with water contents between 10 and 20%, where no definite conclusions can be drawn.

DISCUSSION

A relatively rapid decay of washed *S. marcescens* of water content around 30% has been shown to occur whether the cells are stored in air or in vacuum, and whether or not they have previously been frozen or freeze-dried. The actual rates of decay within this "critical" zone of water content vary somewhat with experimental conditions; air seems to offer some degree of protection.

At very low water contents (0.2 to 7%) a different phenomenon is observed. Here, survival is favored by storage in absence of air; air causes decay at a rate comparable to that observed in the "critical" zone.[5]

The phenomena described here seem to be different from those observed with other types of air-borne microorganisms by Dunklin and Puck (1948), with their uniformly low rate of decay over a wide humidity range in absence of dissolved substances in the suspending medium. This is readily seen when the data are referred to a common variable, the equilibrium RH. Conversion from water content to RH can be made for washed *S. marcescens* by using the water sorption data of J. B. Bateman, C. L. Stevens, W. B. Mercer and E. L. Carstensen (*personal communication*, 1961). Approximate decay constants k for *S. marcescens* dehydrated at room temperature and exposed to air are plotted against RH in Fig. 6, together with values for air-borne type I pneumococci estimated roughly from Dunklin and Puck (1948). Comparison of Fig. 5 and Fig. 6 brings out the fact that the "critical" water phenomenon studied in the present experiments occurs within a narrow range of humidities between 90 and 100% scarcely accessible in aerosol studies with sufficient discrimination to resolve any steeply humidity-dependent variation of decay rates. The data of Dunklin and Puck (1948), on the other hand, cover a broad range of RH (10 to 80%) corresponding to changes of water content (4 to 20%) so small that the measurement of dry weights becomes valueless for determining the effective RH even when the appropriate sorption isotherm is available. It is thus understandable that our viable recovery data within this zone should be erratic,

[5] Additional experiments not reported here suggest that this toxic effect of air may be transient, being no longer observable after about 5 hr of exposure.

suggesting only that there may be humidity-dependent effects here which defy resolution by the method employed. It is in this zone, on the other hand, that aerosol studies with direct measurement of RH are most precise. In short, the "critical" water zone for *S. marcescens* occurs in a region of high humidity not readily controllable in aerosol studies, whereas Dunklin and Puck's data for other microorganisms refer to their behavior within a zone of lower humidities for which no reliable data on *S. marcescens* are available, for the reasons just stated.

In other studies of air-borne bacteria, *S. marcescens* (strain ATCC 274) sprayed from broth suspension, Kethley, Fincher, and Cown (1957) find a flat maximum in the decay rate at RH 40 to 50%. The maximum was less pronounced when other cells of *S. marcescens* (strain unspecified) were sprayed from phosphate buffer, ionic strength 0.002, pH 5.8 to 6.4 (Ferry, Brown, and Damon, 1958), although the absolute rates were high (5 to 10 hr^{-1}), suggesting a toxic effect of the inorganic ions. More recently, Dimmick (1959) has noted a sharp maximum in the rate of loss of viable *S. marcescens* in aerosol form when sprayed from phosphate buffer at RH 45 to 60%, the position and height of the maximum varying markedly with buffer concentration. According to Webb (1959) quite different RH effects are seen with cells grown in absence of NaCl and repeatedly washed in glass distilled water before spraying; the death rate follows a sigmoid curve with a rapid decrease between 60 and 70% RH. This interesting observation suggests that the confused picture obtained from the literature on the aerosol survival of cells as a function
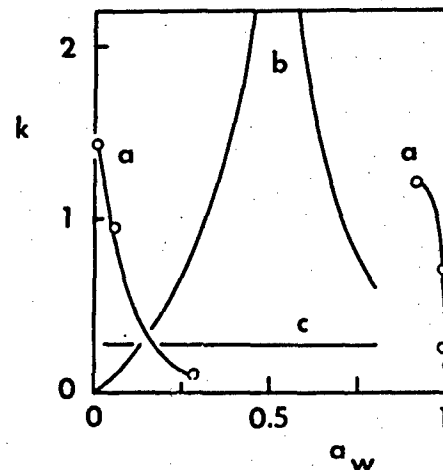

FIG. 6. *Attenuation rate "constants" k in hr^{-1} for bacteria, plotted as a function of equilibrium relative humidity a_w (= RH/100). Curves a represent the data presented in Fig. 5 of this paper for damp Serratia marcescens stored in air. Curves b are based on values estimated from the results of Dunklin and Puck (1948, Fig. 2) for pneumococci sprayed into air from suspension in broth. Line c is for washed pneumococci sprayed into air (Dunklin and Puck, 1948, Fig. 6).*

of RH may arise from failure to control sufficiently the chemical environment. Three contrasting states of the resting *S. marcescens* cell might be envisaged: one dominated by the presence of gross amounts of extracellular chemicals, giving rise to very large humidity-dependent effects; another, in partly washed cells, dominated by toxic substances of intracellular origin insoluble at RH values below about 90%; and a third, in cells which have been washed free of toxic intracellular substances and which exhibit the behavior described by Webb.

The pronounced toxicity of air at very low humidities has not been noted in the literature on *S. marcescens* aerosols; the data of Kethley et al. (1957) extend only to 20% RH, whereas in those of Ferry et al. (1958) the effect may be masked by the toxicity of the phosphate medium. The conclusion of Wells and Zappasodi (1942) that considerable dehydration is unfavorable for the survival of group C β-streptococci depends upon their unwarranted assumption that the effects of very low concentrations (0.05 to 0.3 mg per liter) of propylene glycol are due to a "dehydrating" effect.

The probable existence of zones of instability which cannot readily be demonstrated in conventional aerosol studies shows the need for new techniques. At extremely high humidities, small but decisive changes are accompanied by evaporation or condensation of large amounts of water existing in and around the cell in the form of an aqueous solution whose concentration is very sensitive to small changes of RH. Under these circumstances, it is necessary either to measure water content or to develop refined methods for temperature and RH control. This requirement almost prohibits the use of gross aerosols and leads to the consideration of simulated or semimicroscopic aerosols. At humidities below about 80%, humidity control is the method of choice, whether true or simulated aerosols are used; this is the region of water sorption, in which a given change of RH brings about a change of water content, too small to be measured with precision.

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