Effect of 3% Hydrogen Peroxide on the Viability of *Serratia marcescens*

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Received for publication 1 December 1965

ABSTRACT

CAMPBELL, JACK E. (University of California, Berkeley), AND R. L. DIMMICK. Effect of 3% hydrogen peroxide on the viability of *Serratia marcescens*. J. Bacteriol. 91:925-929. 1966.—Populations of *Serratia marcescens* were exposed to 3% H_2O_2 at temperatures from 0 to 20 C. The reaction appeared to follow an Arrhenius plot, but variable numbers of diminutive colonies were found after cell numbers started to decrease. Colony numbers varied on different sampling media and increased when additional incubation was imposed. The overall reaction was sensitive to age of culture, and growth capabilities of treated samples varied with time of treatment, especially during times when no loss of viability was noted. Catalase activity per cell did not correlate with changes in sensitivity; iron added to growth medium increased catalase activity and decreased sensitivity, but not in the same manner. Although the fundamental reaction is presumably molecular in nature, present methods of viability assay measure more than single events and are not suitable for these studies.

One end product of metabolism in aerobic bacteria is presumed to be hydrogen peroxide (4), which the cell could convert to water by the action of catalase; in high concentrations, however, H_2O_2 is bactericidal. The mechanism of disinfectant action is not known, but excess amounts of H_2O_2 are believed to oxidize some vital internal cell constituent (6). Cellular catalase could protect cells by destroying external H_2O_2 . Initially, we sought information on the thermodynamics of the disinfectant reaction. We investigated the influence of temperature, iron in the growth medium, culture age, and catalase activity on the disinfectant action of 3% hydrogen peroxide. We conclude that the overall reaction does not conform to simple kinetic theory.

MATERIALS AND METHODS

Serratia marcescens, strain 8UK, was grown at 31 C in a chemically defined medium described by Dimmick (2). A 1-ml amount of a sterile solution of ferric ammonium citrate containing 1 mg/ml of iron was sometimes added per liter of growth medium. Data shown in Fig. 1, 2, and 3 were obtained when "house" distilled water from a block-tin still was used. The water was stored in a stainless-steel tank and was delivered through plastic pipe with stainless-steel valves. Data in the remaining figures were obtained with use of water redistilled in a Corning AG-2 all-glass unit.

Unless noted, samples of a single, third-passage

culture of appropriate age were centrifuged, and the cells were suspended in distilled water or supernatant fluid. To provide a relatively constant mass "demand," suspensions were standardized to a total cell mass of 230 Klett units (KU) by a Klett-Summerson photometer and no. 42 filter. This mass was equivalent to approximately 5×10^8 organisms per milliliter in a mature culture. The hydrogen peroxide used was a fresh 1:10 dilution (v/v) of "Superoxol" (30% H_2O_2 ; Merck & Co., Inc., Rahway, N.J.) in distilled water.

To test disinfectant activity, 1 ml of a standardized bacterial suspension was added to 99 ml of 3% H_2O_2 contained in a 250-ml flask immersed in a temperature-controlled water bath. At appropriate intervals, 1-ml samples were mixed with 9 ml of a 1:1,000 dilution of catalase (Nutritional Biochemicals Corp., Cleveland, Ohio; technical grade) to neutralize residual peroxide. Catalase was shown not to affect viability of untreated cells at this concentration. Assay of colony-forming units was done by dropping 0.1-ml quantities of aqueous, 10-fold serial dilutions from a calibrated orifice onto the surface of plates containing either chemically defined medium agar or Trypticase Soy Agar (BBL). Plates were incubated at 31 C.

Growth curves of injured cultures (Fig. 2) were obtained by means of an instrument of our own construction. In brief, a fraction collector was modified to permit right-angle scattered light from suspended particles to be detected by a self-generating photocell as each of 60 tubes containing 5 ml of medium passed sequentially through the unfiltered beam from a microscope lamp. Photocell output was charted on a

servorecorder. Growth curves of untreated cultures were always similar to the curve labeled O in Fig. 2. The time of incubation required to yield an arbitrary (20 mv) signal level correlated with the logarithm (2 through 8) of the number of viable cells in the inoculum, and curves from replicate tests of a given inoculum (0.1 ml) varied no more than $\pm 15\%$. This method represented an arbitrary, simultaneous, and sensitive measurement of the growth capacity of numerous samples, and could be used to estimate the number of viable cells in the inoculum.

Catalase activity was assayed by mixing 1 ml of the bacterial suspension with 100 ml of 0.001 M H_2O_2 and then determining the residual peroxide concentration at various time intervals by the photometric titanium sulfate method of Eisenberg (3), with use of the reagent modification of Bonet-Maury (1).

RESULTS

Survivor curves at temperatures between 0 and 20 C are shown in Fig. 1. Curves were biphasic, with an initial period of little or no death, followed by an almost logarithmic decay period (a possible multiple-hit reaction). A plot of the logarithm of the specific decay constants of the latter slopes versus $1/T$ was linear.

The validity of such data, however, was com-

FIG. 1. Survival of a 24-hr culture of *Serratia marcescens* grown without added iron, then exposed to 3% H_2O_2 at different temperatures. All survival curves (Fig. 1, 2, 3, 5, 6, and 7) are based on maximal estimates.

plicated by the presence, on both types of agar, of numerous diminutive colonies from samples obtained after colony numbers began to decrease, and the observation that additional colonies often appeared after extended incubation of the plates. We found no evidence that diminutive characteristics were transmissible, so we assumed that the recuperative capacity of cells had been changed by injury. To investigate this further, we determined the growth capabilities of cells surviving various contact times at 20 C (Fig. 2). Relative growth rates of exposed samples were often less, rates were not constant, and maximal growth was lower than those not treated with H_2O_2 . Relative turbidity attained by these cultures at 24 hr, as well as the estimated viability from such measurements, is compared with a corresponding survivor curve in Fig. 3. The number of colony-forming units did not decrease until after about 4 min of contact with H_2O_2 , whereas growth capability changed immediately after contact.

The validity was also complicated by the influence of culture age. The change in catalase activity during culture growth is shown in Fig. 4. Without added iron, and with glass-distilled water, the culture evinced a 4-hr delay before an increase in catalase activity was noted. The activity doubled after 6 hr and then gradually declined. With 1 mg per liter of added iron, the culture showed no delay, and an 8- to 10-hr peak of approximately twice the original activity was evident. Subsequently there was a gradual decline, but activity remained high. Increasing

FIG. 2. Growth (in terms of relative turbidity and without added iron) of samples (the first 10-fold dilution used for viability assay) from a 24-hr culture of *Serratia marcescens* treated with 3% H_2O_2 for the times indicated. All samples were planted within the first 22 min of the noted incubation period. All curves from this test are not shown.

FIG. 3. Survivor curve of *Serratia marcescens* treated with 3% H_2O_2 at 20 C and two estimates of growth capability during treatment. Cells (24-hr-old) were grown without added iron.

FIG. 4. Catalase activity of *Serratia marcescens* as a function of culture age. Inoculum was grown without added iron.

the added iron concentration to 20 mg per liter did not measurably increase the catalase activity. Hence, the iron concentration for maximal activity must be at or below the 1.0 mg per liter level. We did not find an increased growth rate or extent of growth in defined medium when 1.0 mg per liter of iron was added.

The number of colony-forming units constituting a 230-KU standard-mass suspension varied with culture age (Fig. 5). The suspension made from a 4-hr-old culture had the lowest count per milliliter (i.e., cells had reached a maximal size); a mature culture, the highest.

Peroxide sensitivity of *S. marcescens* changed during the growth cycle. The number of colony-forming units recovered after 2-, 4-, and 6-min exposure to 3% H_2O_2 declined during the first 4 hr of growth to a minimal value at approximately 5 hr (Fig. 6). As the culture aged, resistance increased, reaching a maximum at 16 hr. As Fig. 6 shows, recovery on Trypticase Soy Agar was higher than on defined agar in almost all instances. The greatest difference occurred during the 4- to 6-hr period.

With added iron in the growth medium, survival characteristics as a function of age were different from cultures grown without iron (Fig. 7), but older cultures were similar to the one

previously noted (Fig. 3). Resistance was still minimal at 4 to 6 hr, but not as low as without added iron. Thereafter, resistance increased, reaching a maximal value at 16 hr, and then remained constant. Differences in recovery between two sampling media were not as great as before but were predominantly opposite; use of a defined plating medium resulted in either higher or equal recovery. Addition of iron to the medium in which cells were grown increased both cell survival and catalase activity; it did not affect growth rate or final cell concentration.

Combining data from Fig. 4, 5, 6, and 7 revealed additional information (Fig. 8). Cells grown with or without iron attained maximal size and maximal sensitivity simultaneously, but not maximal catalase activity. The sequence of events was, first, maximal size, then sensitivity, followed by maximal catalase activity per cell.

DISCUSSION

We believe that formation of diminutive colonies is caused by a multiplicity of cellular injuries that prevent normal colony development (2). Although damage to the genetic constituents of the cell might have occurred, we were unable to demonstrate it by the colonial transfer method.

Growth curve data (Fig. 2) may be interpreted

FIG. 5. Colony-forming units of *Serratia marcescens* in a standard suspension as a function of culture age. A typical growth curve is superimposed for comparative purposes. Inoculum was grown without added iron.

FIG. 7. Survival (as a function of culture age and of time of exposure to 3% H_2O_2) of *Serratia marcescens* grown in defined medium, with glass-distilled water, containing 1.0 mg of iron per liter. Inoculum was grown without added iron.

FIG. 6. Survival (as a function of culture age and of time of exposure to 3% H_2O_2) of *Serratia marcescens* grown in defined medium with glass-distilled water and no added iron. Inoculum was grown without added iron.

FIG. 8. Relative catalase activity per cell as a function of culture age. Included are the times when cells reached maximal size or maximal sensitivity to H_2O_2 .

in two ways: first, that after an indefinite lag, some surviving cells started to grow slowly, but their doubling time decreased as the culture grew (this implies that growth rate was limited initially

by a repairable injury, but cell division was not completely prevented); second, that the lag time of some cells which were going to grow was lengthened, but after the first division the rate was normal (this implies a repairable injury which prevented initial cell division). Of course, there is an environmental difference between growth on agar surfaces and in liquid menstruum; consequently, the same mechanisms of growth impairment cannot be assumed for both instances. We can state with certainty that we found diminutive colonies only with injured cultures, and we found aberrant growth curves only with injured cultures. The two environments were undoubtedly selective, as shown by the difference (Fig. 3) during the first 4 min of treatment. During this time, there was no loss of viability and no diminutive colony forms, but growth in liquid medium was impaired. Therefore, either at least two types of injury were involved or the injury for a given cell was progressive; the apparent 4-min lag in the survivor curve was not caused by failure of H_2O_2 to contact some responsive cellular site.

The difference between the number of colonies on two different sampling media, greatest as cells approached the age of maximal sensitivity, can be explained by assuming that some cells grown in medium without added iron were sensitive because of iron deficiency. The organic sampling medium allowed a greater number of cells to grow by furnishing additional iron. Conversely, cells grown with iron were in a more "normal" state, assuming that optimal catalase production is a positive survival mechanism. The organic medium was a change, but not a significant improvement. In fact, during the adaptive, early growth phase (0 to 6 hr) the change was detrimental (Fig. 7).

McCarthy and Hinshelwood (5), using *Aerobacter aerogenes*, found changes in catalase activity during culture growth to be influenced by pH, but our medium remained near pH 7.0 during the entire growth cycle. Therefore, the change in activity (Fig. 8) was caused by some

other factor; possibly a greater enzyme turnover or increased contact between peroxide and catalase in the cell (permeability) occurred as the culture aged. The difference in cell numbers between "young" and "old" standardized cultures was several orders of magnitude less than the difference in survival capacity, and noted events (Fig. 8) were out of phase. Certainly, neither cell numbers, cell size, nor catalase activity was directly related to survival ability.

There is no doubt in our minds that the fundamental reaction between peroxide and some sensitive parts of the cell is a molecular event, possibly genetic, but there is interference (repair of injury of an indefinite extent) between the event and the measurement of that event in terms of colony formation. Any single criterion of "life," especially colony formation (when considered an all-or-none indicator), is not always an adequate measurement of the influence of an adverse environment on the resulting cellular growth capacity.

ACKNOWLEDGMENT

This investigation was sponsored by the Office of Naval Research under a contract with the Regents of the University of California.

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