Factors Affecting Comparative Resistance of Naturally Occurring and Subcultured *Pseudomonas aeruginosa* to Disinfectants

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A strain of *Pseudomonas aeruginosa* was isolated in pure culture from the reservoir of a hospital mist therapy unit by an extinction-dilution technique; its natural distilled water environment was used as a growth and maintenance medium. After a single subculture on Trypticase soy agar, the strain showed a marked decrease in resistance to inactivation by acetic acid, glutaraldehyde, chlorine dioxide, and a quaternary ammonium compound when compared with naturally occurring cells grown in mist therapy unit water. The following factors were observed to affect the relative resistances of naturally occurring and subcultured cells of the *P. aeruginosa* strain: (i) temperature at which the cultures were incubated prior to exposure to disinfectants, (ii) growth phase of the cultures at the time of exposure to disinfectants, (iii) nature of the suspending menstruum for disinfectants, and (iv) exposure to fluorescent light during incubation of inocula prior to testing. The applied significance of these findings may alter the present concepts of disinfectant testing as well as routine control procedures in the hospital environment.

The increasing incidence of nosocomial infections with *Pseudomonas aeruginosa* and other *Pseudomonas* species has focused attention on more effective methods of controlling these organisms in the hospital environment. Reservoirs of infection have been found in contaminated urological instruments (10, 14), blood pressure monitoring systems (15), various types of inhalation therapy equipment (7, 9, 16, 17, 19), and also in disinfectant solutions used to decontaminate such equipment (5, 6, 10, 12, 21). These gram-negative organisms are not only able to multiply but also maintain high population levels in distilled water (7, 8, 13) as well as in certain disinfectants (1, 5, 6). Although heat treatment (6, 14, 19) and gaseous sterilization (9) have proven effective as control measures, many types of equipment and supplies necessitate the use of chemical disinfectants.

A variety of techniques for measuring the efficacy of disinfectants have been evaluated in the laboratory, and official standards (4) now adopted include rigid specifications with respect to the subculturing of test organisms, the types of nutrient media used, and growth temperatures employed. These standards are based, in part, on the assumption that the behavior of a microorganism in a controlled laboratory environment should reflect that organism's response in its natural ecological niche.

We reported previously (8) on the isolation of a strain of *P. aeruginosa* from the distilled water reservoir of a hospital mist therapy unit (MTU). Filter-sterilized MTU water (MTUW) was employed as a "growth" medium, and a pure culture of *P. aeruginosa* was isolated by an extinction-dilution technique. Since it was not subcultured on conventional laboratory media but rather isolated and maintained in its original ecological niche, it was designated as a "naturally occurring" strain. Experiments were designed to compare the growth characteristics of the naturally occurring strain in MTUW with cells of the same strain subcultured once on Trypticase soy agar (TSA; Baltimore Biological Laboratory) and then introduced either into MTUW or buffered distilled water (BDW; 2). The naturally occurring cells of *P. aeruginosa* showed no lag phase and grew rapidly in either system. Subcultured cells did
not survive in BDW, and in MTUW initial numbers were reduced by three logs before exponential growth occurred.

The results of these studies suggest that the characteristic behavior of an organism in its natural environment may in fact be markedly altered during isolation, purification, and maintenance on laboratory culture media. Since use-dilutions of disinfectants are routinely determined by tests employing laboratory strains which have been subcultured many times, this may explain some of the discrepancies between observed in vitro results and the actual use of disinfectants in applied situations.

The purpose of this study was to assess the comparative resistances of naturally occurring cells of a strain of P. aeruginosa and cells obtained from subculturing on TSA one time to inactivation by various disinfectants and to examine some of the factors affecting their responses.

MATERIALS AND METHODS

Disinfectants and neutralizers. The quaternary ammonium compound (QAC) was a commercial detergent sanitizer (Mikro-Quat; Economics Laboratory, Inc., St. Paul, Minn.) containing 9.0% alkyl (50% C14, 40% C13, 10% C12) dimethylbenzyl ammonium chloride and 0.4% triiodium ethylenediamine-tetraacetic acid as the active ingredients. QAC was neutralized by dilution in sterile skim milk containing Tween 80 (Polyoxbate 80; Hilltop Research Inc., Miamiville, Ohio). Glutaraldehyde, a commercial preparation (Cidex; Arbrook, Somerville, N.J.) containing 2.0% of active ingredient was neutralized by sodium bisulfite. Chlorine dioxide, in a commercial preparation (Oxine; Lily Products Co., Phoenix, Ariz.) containing 2.0% of active ingredient, was neutralized by sodium thiosulfate. Acetic acid (analytical reagent; Mallinckrodt, St. Louis, Mo.) was neutralized by dilution in BDW.

Preparation of cell suspensions. Distilled water obtained from the reservoirs of hospital mist therapy units was pooled, incubated at room temperature for several days to simulate hospital conditions which would allow growth of gram-negative contaminants (ripened), and then sterilized by membrane filtration (Millipore Corp., 0.22 μm GA type). "Unripened" distilled water from the stock bottles used to fill the reservoirs also was obtained and was immediately filter-sterilized. These waters (MTUW) were used both as growth and maintenance media for the naturally occurring strain of P. aeruginosa previously isolated in MTUW (8) and as suspending menstrua for disinfectants.

A 15-ml amount of sterile MTUW was inoculated with 0.1 ml of naturally occurring P. aeruginosa maintained in MTUW. Cultures were incubated at 25 C for 24 and 112 hr to obtain log- and stationary-phase cells, respectively, and at 37 C for 48 hr to obtain stationary-phase cells. Although test cultures were routinely incubated in the dark, additional experiments were designed to test the effect of exposure to light during growth on subsequent resistance levels. Artificial lighting was provided by a bank of eight 20-w fluorescent lights (Westinghouse; cool-white high-output) in a PsycoTherm incubator shaker (New Brunswick Scientific Co., New Brunswick, N.J.) 25 cm above the growing cultures. After incubation, 10 ml of the MTUW growth suspension was added at room temperature (25 C) to the reaction flask (250 ml, Erlenmeyer) to yield a final concentration of naturally occurring cells of approximately 10^4/ml in 109 ml of MTUW.

For the preparation of subcultured cells, a 0.1-ml inoculum from the maintenance culture of naturally occurring cells was spread over the surface of a TSA plate. Cultures were incubated at 25 C for 18 and 48 hr for log- and stationary-phase cells, respectively, and at 37 C for 24 hr for stationary-phase cells. Growth was aseptically harvested and washed twice in sterile BDW. The final BDW suspension was adjusted turbidimetrically so that 1.0 ml added to the reaction flask yielded a final concentration of subcultured cells of approximately 10^4/ml in 109 ml of MTUW.

Susceptibility tests. From the reaction flasks containing the naturally occurring and subcultured cell systems in MTUW 10 ml were removed to serve as controls. One milliliter of the disinfectant, diluted in MTUW, was then added at room temperature to each of the reaction flasks to yield final concentrations and pH values as follows: QAC, 2.5 ppm, pH 6.9; glutaraldehyde, 31 ppm, pH 7.5; chlorine dioxide, 67 ppm, pH 6.5; and acetic acid, 0.25%, pH 3.1. Although the QAC and glutaraldehyde preparations were less than the recommended use dilution strengths, these concentrations were shown in preliminary tests to inactivate the cell systems at rates optimal for comparative studies. At each subsequent time interval, samples from the control and disinfectant reaction flasks were removed, added to sterile tubes containing appropriate quantities of neutralizing agent, and plated in duplicate in TSA supplemented with 0.5% KNO3 to enhance subsurface growth. Colony counts of the control suspensions showed no decline in the numbers of viable organisms, and survivor curves were plotted from colony counts of the control and test series after 48 hr of incubation at 37 C. All data points presented in this paper are mean values of replicate plates at dilutions that resulted in plate counts of between 30 and 300 colonies. The figures represent typical results based on multiple tests of each of the variables studied.

RESULTS

Effect of growth temperature. The temperature at which test suspensions of naturally occurring and TSA-subcultured cells of P. aeruginosa were incubated prior to exposure to disinfectants appeared to affect subsequent resistances. Naturally occurring cells incubated in MTUW at 25 C were markedly more resistant to 0.25% acetic acid than TSA-sub-
cultured cells grown either at 25 or at 37 C (32.0% survivors versus 0.5 and 0.02%, respectively, in 90 min; Fig. 1). However, naturally occurring cells incubated at 37 C showed a notable increase in the rate of inactivation, with only 0.1% survivors at 30 min and no detectable survivors at 60 min. TSA-subcultured cells grown at 25 C showed somewhat greater resistance to 0.25% acetic acid than cells grown at 37 C. Increased resistance to chlorine dioxide, QAC, and glutaraldehyde was also observed with subcultured cells grown at 25 C as compared with 37 C, but these differences did not appear to be significant.

**Effect of growth phase.** Cells of naturally occurring *P. aeruginosa* grown in ripened MTUW at 25 C and those subcultured a single time on TSA at 25 C were exposed to disinfectants in the log and stationary phases of their respective growth cycles. Results obtained with exposure to glutaraldehyde and 0.25% acetic acid are shown in Table 1. Stationary-phase cells of the naturally occurring organism showed much greater resistance to 0.25% acetic acid than log-phase cells. The growth phases of TSA-subcultured cells, however, showed essentially no difference in their susceptibilities to acetic acid, and in either phase were more susceptible than the stationary-phase cells of the naturally occurring organism. With glutaraldehyde, however, log-phase cells of the naturally occurring organism unexpectedly showed greater resistance than stationary-phase cells.

### Table 1. Effect of growth phase on resistance of naturally occurring and subcultured cells of *Pseudomonas aeruginosa* exposed to acetic acid and glutaraldehyde

| Length of exposure (min) | Survival (%) |
|--------------------------|--------------|
|                          | Acetic acid (0.25%) | Glutaraldehyde (31 ppm) |
|                          | Stationary phase | Log phase | Stationary phase | Log phase |
| Naturally occurring cells* |               |           |                 |           |
| 30                       | 61.2          | 12.8      | 18.0            | 9.0       |
| 60                       | 48.1          | 4.3       | 0.01            | 0.7       |
| 90                       | 33.1          | 1.6       | NDs*            | 0.1       |
| Subcultured cells*       |               |           |                 |           |
| 30                       | 37.0          | 38.5      | 1.7             | 0.01      |
| 60                       | 7.9           | 6.9       | 0.004           | NDs       |
| 90                       | 2.3           | 0.7       | NDs             | NDs       |

* Naturally occurring cells cultured in ripened mist therapy unit water at 25 C.
* No detectable survivors.
* Subcultured cells grown on Trypticase soy agar at 25 C.

A marked tailing effect was seen, and viable organisms were recovered even after 120-min exposure. In the subcultured cell system, stationary-phase cells showed slightly greater resistance to glutaraldehyde than log-phase cells but were less resistant than either stationary- or log-phase cells of the naturally occurring strain.

**Nature of suspending menstruum.** Figure 2 shows the comparative resistances of naturally occurring and subcultured cells of *P. aeruginosa* exposed to disinfectants in ripened and unripened MTUW. With ClO₂ (Fig. 2A), both naturally occurring and subcultured cells showed markedly greater resistances in ripened MTUW than in unripened MTUW. In both suspending menstrua, however, the naturally occurring cells were only slightly more resistant than subcultured cells. With QAC (Fig. 2B) and glutaraldehyde (Fig. 2C), the naturally occurring cells not only showed greater resistance in ripened MTUW, but in either ripened or unripened MTUW were more resistant to both disinfectants than were subcultured cells in either suspending menstruum. Subcultured cells showed much greater resistance to QAC in ripened than in unripened MTUW, but there was essentially no difference when they were exposed to glutaraldehyde in ripened and unripened MTUW.

Figure 3 shows the comparative resistances
of naturally occurring and subcultured cells exposed to 0.25% acetic acid both in ripened and unripened MTUW and in brain heart infusion (BHI) broth (Baltimore Biological Laboratory). The organisms were first cultured in MTUW and on TSA, as above, and then exposed to acetic acid in MTUW and BHI in the reaction flasks. Again, the naturally occurring cells in either ripened or unripened MTUW were considerably more resistant than subcultured cells in either suspending menstruum. However, virtually no inactivation was obtained with either naturally occurring or subcultured cells exposed to 0.25% acetic acid in BHI. When the concentration of acetic acid in BHI was increased to 2.5% both naturally occurring and subcultured cells showed no survivors after 15 min exposure.

**Effect of fluorescent light.** Table 2 shows results obtained with naturally occurring cells exposed to disinfectants after growth in the presence and absence of fluorescent light. Resistance to QAC was markedly and immediately affected; a 5-min exposure produced 99.6% inactivation of light-grown cells, whereas a 90-min exposure inactivated only 98.8% of dark-grown cells. Altered resistance to acetic acid and ClO₂ was also evident in light-grown cells, although the rates of inactivation were somewhat lower than those obtained with QAC. Resistance to glutaraldehyde did not appear to be affected by exposure of naturally occurring test cells to fluorescent light. In similar experiments with subcultured cells, resistance to ClO₂ and QAC was only slightly decreased after exposure to fluorescent light during growth, and no differences were noted when 0.25% acetic acid and glutaraldehyde were tested.

**DISCUSSION**

Past studies on the response of *Pseudomonas* species to disinfectants have shown discrepancies between in vivo and in vitro tests. For example, Griebel et al. (9) reported that 2.0% acetic acid failed to eliminate *P. aeruginosa* from humidifier units in a pulmonary disease ward. However, in vitro studies of Rhoades and Short (18) demonstrated bactericidal activity of 1.8% acetic acid against *P. aeruginosa* in BHI broth, and Hedberg and Miller (11) demonstrated inactivation of aqueous suspensions of broth-grown *P. aeruginosa* by 1.0% acetic acid.

In studies of other disinfectants, glutaraldehyde (2%) failed to disinfect ultrasonic nebulizers heavily contaminated with *Pseudomonas* species (16), but it was shown in vitro to rapidly inactivate *P. aeruginosa* in concentrations as low as 0.05% (20). Lowbury (12) reported the isolation of *P. aeruginosa* from hospital stock bottles of 1.0% cetrimide solutions, but peptone-water cultures of these strains failed to

![Fig. 2. Effect of suspending menstruum on resistance of naturally occurring and subcultured cells of *Pseudomonas aeruginosa* to disinfectants. A, Chlorine dioxide (67 ppm); B, quaternary ammonium compound (2.8 ppm); C, glutaraldehyde (31 ppm).](image-url)
survive when introduced into even 0.015% cetrimide solutions. Basset et al. (5) reported that a strain of <i>P. multivorans</i> (cepacia) detected in stock bottles of 1:30 Savlon (0.05% chlorhexidine, 0.5% cetrimide) could survive and multiply after repeated transfers in 1:30 Savlon but became sensitive to 1:320 Savlon after subculture onto nutrient media.

Association of Official Agricultural Chemists official tests (4) include specifications as to the types of maintenance and subculture media to be used in the preparation of test cultures of <i>P. aeruginosa</i>, the number of subcultures to be made immediately prior to testing (at least three), and the growth temperature (37 C) and growth phase (stationary) at which test organisms are to be cultured and harvested. However, in the studies presented here comparing the resistances of naturally occurring and subcultured cells of a strain of <i>P. aeruginosa</i> to acetic acid, QAC, glutaraldehyde, and ClO₂, results showed that cells of naturally occurring <i>P. aeruginosa</i> subcultured even once on an enriched medium (TSA) at 37 C did not begin to approach the degree of resistance demonstrated by cells grown in their natural environment (MTUW). Even when subcultured at 25 C, a temperature more closely approximating that of the natural environment in the hospital, the subcultured cells, although showing slightly more resistance than cells at 37 C, still fell far short of reflecting the resistance of the naturally occurring organism. Whether grown at 37 or 25 C, cells of <i>P. aeruginosa</i> appear to undergo some type of alteration during subculture which subsequently renders them more susceptible to inactivation by chemical agents. Whether this involves a change in the chemical composition of the cell, altered cell permeability, or other factors is not yet known.

Similarly, differences in the growth-phase response of naturally occurring <i>P. aeruginosa</i> and TSA-subcultured cells to disinfectants were observed. The degree of resistance of naturally occurring <i>P. aeruginosa</i> to various disinfectants appeared to depend, in part, on the physiological age of the naturally occurring cells at the time of exposure. With acetic acid, stationary-phase cells of the naturally occurring organism were more resistant than log-phase cells, but with glutaraldehyde just the opposite was noted. With cells subcultured once on TSA, however, the resistance of sta-

![Fig. 3. Effect of suspending menstruum on resistance of naturally occurring and subcultured cells of Pseudomonas aeruginosa to 0.25% acetic acid.](image)

| Length of exposure (min) | Survival (%) |
|-------------------------|--------------|
|                          | Quaternary ammonium compound | Chlorine dioxide | Acetic acid | Glutaraldehyde |
|                          | Dark-grown | Light-grown | Dark-grown | Light-grown | Dark-grown | Light-grown | Dark-grown | Light-grown |
| 5                       | 20.0       | 0.4        | 91.0       | 84.0       | 98.0       | 54.0       | 64.0       | 60.0       |
| 15                      | 15.8       | 0.1        | 30.0       | 16.0       | 85.0       | 17.5       | 24.7       | 20.0       |
| 30                      | 3.7        | NDS        | 1.6        | 0.08       | 73.5       | 5.9        | 1.0        | 2.2        |
| 60                      | 1.4        | NDS        | 0.06       | NDS        | 52.9       | 0.35       | 0.04       | 0.06       |
| 90                      | 1.2        | NDS        | 0.003      | NDS        | 39.2       | NDS        | 0.002      | NDS        |

* Naturally occurring cells grown in unripened MTUW at 25 C for 112 hr either in the absence of light or exposed to fluorescent light.
* No detectable survivors.
tionary-phase cells was only slightly greater than that of log-phase cells with either acetic acid or glutaraldehyde. Moreover, the TSA-subcultured cells in either stationary or log phase showed less resistance than cells of the naturally occurring organism. These results, whether reflecting differences in the chemical makeup or physiological capacity of naturally occurring as opposed to subcultured cells, may nevertheless explain the failure of some disinfectants when used in applied situations, even though previously shown to be effective in vitro.

Since hospital environments, like populations of organisms, do not remain static, modifications in the ecological niche of a naturally occurring organism may also contribute to altered responses to disinfectants. Purportedly effective in laboratory use dilution tests, disinfectants may, in fact, be used in environments considerably modified by variations or even lapses in hospital procedures and practices (3).

For example, faulty technique in the cleaning and preparation of glassware used as stock containers for distilled water or disinfectants which result in the continued presence of trace nutrients and bacterial inocula, the introduction of volatile materials into distilled water in the supply lines or storage tanks of the stills, or lack of aseptic technique in the filling of distilled water reservoirs in inhalation therapy equipment may all contribute in the chain of events producing altered environments suitable for the rapid growth of naturally occurring organisms. Thus, the introduction of *P. aeruginosa* into distilled water in MTU reservoirs which contain even trace amounts of dissolved organic nutrients and other growth substances has been shown to result in rapid multiplication of the organism (8). The MTU environment consequently presents an entirely different operational milieu for disinfectants. The presence of presumably altered types and amounts of organic and inorganic materials as well as changes in pH may lead to adsorption, alteration, or inactivation of disinfectants, significantly reducing recommended effective concentrations. Moreover, substandard preparations of working solutions from stock concentrates of disinfectants, contamination of solutions, failure to replace solutions which have deteriorated upon standing, or even dilution of residual disinfectant by routine refilling of distilled water reservoirs may all cumulatively modify the intensity of response of a particular species to any disinfectant, sometimes resulting in gross breakdown of effective decontaminating procedures.

As reported here, the simple alteration of a natural MTUW environment by growth of contaminating gram-negative organisms (ripened MTUW), even though the contaminants themselves were subsequently removed by filtration, markedly increased the resistance of naturally occurring cells of *P. aeruginosa* to QAC and ClO₂. The TSA-subcultured cells, although exhibiting slight increases in resistance when exposed to these disinfectants in ripened versus unripened MTUW, showed no parallel to the response of the naturally occurring cells. Similar results were obtained in exposing naturally occurring and subcultured cells to 0.25% acetic acid in ripened and unripened MTUW (pH 3.1 and 3.2, respectively). Moreover, 0.25% acetic acid in BHI broth (pH 5.0), simulating the action of disinfectants in much more complexly altered or contaminated hospital environments, failed to inactivate naturally occurring cells; subcultured cells showed slight inactivation. However, increasing the concentration of acetic acid to 2.5% in BHI broth (pH 3.7) produced such rapid inactivation of both cell systems that differences between naturally occurring and subcultured cells could not be measured. Nevertheless, these results do indicate that the effectiveness of acetic acid as a disinfectant is not merely a function of pH and that inactivation of naturally occurring populations by disinfectants can be altered considerably by the nature of the suspending menstrum as it may exist in the natural environment.

Many other factors obviously operate to modify hospital environments, including physical factors such as changes in relative humidity, varying air movement through air conditioning or recirculating systems, and germicidal properties of natural or artificial radiations, any of which may change the type and density of microbial populations present. Studies reporting germicidal effects of radiation on microorganisms have been largely concerned with wavelengths below 40 nm, although artificial (fluorescent) lighting at visible wavelengths of 45 to 60 nm has been shown to affect viability of cells (22). As was reported here, a naturally occurring strain of *P. aeruginosa* previously resistant to acetic acid, QAC, and ClO₂ showed subsequently marked increases in rates of inactivation when exposed to these disinfectants after growth under fluorescent light. Although such results must be interpreted with caution, it is not unreasonable to consider that the effectiveness of disinfectants used in hospitals might be related either to variations in the inherent sus-
ceptibilities of naturally occurring microbial populations to radiation effects or to variations in the length of exposure and the intensity of visible light radiations in the hospital environment. In some situations, different designs and locations of equipment might allow visible light to enhance the antimicrobial properties of disinfectants.

From these findings it would appear that the constant fluctuations which occur in natural environments in the hospital, coupled with disparities between stated principles of asepsis and actual hospital practices, can greatly compound the problems attendant to control of hospital infections. Further, when results obtained solely with laboratory-adapted, subcultured cell systems are extrapolated to naturally occurring populations in the hospital environment, such methodology could introduce significant error in evaluating the efficacy and use of disinfectants.

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