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Effect of Physiological Age and State on Survival of Desiccated *Pseudomonas aeruginosa*

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Cells of *Pseudomonas aeruginosa* harvested after 6 hr, 24 hr, 7 days, 33 days, and 120 days of incubation at 36 C varied in their resistance to the deleterious effects of desiccation. The longest survival period observed was for cells from cultures incubated for 7 days. Cells of the exponential growth phase were the most susceptible. The addition of extracellular slime from 7-day cultures to suspending medium did not increase survival of cells from the exponential growth phase. No marked differences in survival time were observed for cells of similar physiological age from cultures incubated at 20, 36, and 42 C. In comparison with the laboratory strain, *P. aeruginosa* OSU 64, used in this study, strains isolated from natural habitats and subjected to a minimum of manipulation in the laboratory were not better adapted to withstand the stress of desiccation.

The infrequent recovery of *Pseudomonas aeruginosa* from dust, dry surfaces, and other types of bacterial habitats lacking moisture suggests that the organism cannot withstand the deleterious effects of desiccation. Extrinsic conditions, including the manner of drying, rate and extent of water loss, type of suspending media, and relative humidity (RH) and nature of the gaseous microenvironment influence survival by retarding or enhancing the lethal action of dehydration during and after desiccation. In addition, resistance to desiccation is influenced by the physiological age and state of the bacterial cell.

In previous studies concerned with survival of dried *P. aeruginosa*, data have been accumulated with 18- to 24-hr cultures. When aqueous suspensions were dried on cover slips at room temperature, only a small fraction of the initial population was viable after 100 min (6). With cells suspended in nutrient broth and dried at room temperature in an atmosphere of approximately 50% RH, a small number of viable organisms were recovered after 24 hr (8). A much longer survival period, up to 150 days, was observed with *P. aeruginosa* of this age deposited on filter paper and dried at room temperature (2).

Survival of *P. aeruginosa* cells with different physiological ages and states has not been investigated previously. The present study was undertaken to determine whether resistance to the effects of drying is influenced by strain differences, age of cells, and temperature during growth. Relatively small numbers of organisms were exposed in each trial, to prevent formation of agglomerations in which the innermost cells would be protected against drying by surrounding cells and cellular debris. Desiccation of small numbers ensures more uniform dehydration of individual cells and indicates more precisely cellular resistance to drying rather than the behavior of a desiccated bacterial population.

MATERIALS AND METHODS

Bacterial strains. Except for determining resistance of organisms recently isolated from natural habitats, the same strain, *P. aeruginosa* OSU 64, was used throughout the study. This strain has been maintained under laboratory conditions in the Department of Microbiology, University of Georgia, for several years and was deposited with the American Type Culture Commission as strain no. 25327. Stock cultures were transferred at 3- to 4-week intervals and stored at 4 C.

Resistance to desiccation was also determined for strains isolated from a wound of a hospitalized patient and from the wash basin and the water faucet in the patient's room. Cells from these strains were desiccated, after a minimum of manipulation in the laboratory, to establish purity of growth.

Media. Brain heart infusion (BHI) broth (Difco) was used for culturing cells exposed to desiccation. Viability of the desiccated organisms was determined by surface plating the recovery liquid on BHI agar fortified with 5% defibrinated sheep blood.

Preparation of seed cultures. Seed cultures for initiating growth of cells subsequently exposed to desiccation were obtained by transferring a small portion of a 24-hr colony cultured on blood agar to a 50-ml DeLong culture flask containing 20 ml of medium. The flask was incubated at 36 C. Aeration of the liquid was accomplished by agitation on a rotary platform shaker. Approximately 0.01 ml of the 24-hr growth was transferred to a second 20-ml volume of culture medium, which was also incubated for 24 hr. This procedure was repeated, and the 24-hr growth of the third transfer was designated as the seed culture.

Preparation of test cultures. In experiments concerned with survival of physiologically young cells, growth was initiated by introducing 2 ml of a 10^{-9} dilution of the seed culture containing 5 to 20 organisms into a 500-ml DeLong flask containing 200 ml of BHI broth which had been warmed to 36 C. The inoculated medium was immediately placed on a rotary shaker and incubated at 36 C for 6 hr. Although multiplication occurred during the incubation period, the appearance of the broth did not change either in clarity or color. Cells from the 6-hr culture were harvested by filtration through a 0.22-μm membrane filter (Millipore Corp., Bedford, Mass.). The filter with entrapped organisms was transferred to a test tube containing 4 ml of filtrate and placed on a wrist-action shaker, and the cells were suspended by shaking for 30 min at approximately 250 oscillations per min. Survival of 6-hr organisms dried in bovine serum was determined with cells recovered from the filter by washing with serum instead of culture broth.

An inoculum of 5 to 20 cells for 200 ml of BHI broth in 500-ml DeLong flasks was also used to initiate growth of test organisms from cultures incubated for 24 hr or longer. Cell populations from these cultures were too large for direct deposition on the drying surface. This necessitated diluting the cultures with the respective spent medium. The desired number of cells was obtained by diluting 1 ml of the culture with cell-free medium prepared by centrifuging approximately 75 ml of the culture and then filtering the supernatant fluid through a 0.22-μm membrane filter.

Drying procedure. Test suspensions of 0.01 or 0.02 ml were deposited on individual squares 1 cm in size and dried over $CaCl_2$ in a desiccator evacuated to 20 in Hg. Drying required approximately 20 min when 0.01 ml of suspension was placed on individual squares and approximately 30 min when the volume was 0.02 ml. As soon as the visible liquid evaporated completely, filtered dry air was introduced into the desiccator until atmospheric pressure was restored. The glass squares were then removed and immediately placed in an atmosphere of 25 C and 53% RH (7).

Enumeration of survivors. Resistance to drying was determined by enumerating viable organisms recovered from two glass squares selected at random immediately before and after drying and at subsequent 24-hr intervals. The glass squares were placed in individual test tubes containing 2 ml of BHI broth and agitated on a wrist-action shaker for 20 min. Four 0.25-ml samples of the recovery liquid were streaked on individual blood agar plates. After the liquid was absorbed, the plates were inverted and incubated at 36 C. Survival of organisms held at 25 C and 53% RH was determined daily until glass squares assayed on 3 or more consecutive days failed to yield viable organisms.

Blood agar plates were incubated at 36 C for 72 hr. Plates showing no growth were incubated for an additional 4 days. The number of survivors recovered from glass squares was expressed in terms of the number of colonies developing on the agar surface.

RESULTS

Viability of dried cells. Cells of *P. aeruginosa* OSU 64 harvested from BHI cultures incubated for 24 hr at 36 C and exposed to drying exhibited a rapid loss of viability (Table 1). All but a small number were irreversibly damaged within 20 min. Further reduction occurred during the next 2 hr, at the end of which time only a small number of survivors were recovered. On days 1 to 4 after desiccation, no viable cells were recovered from glass squares held at 25 C and 53% RH.

Effect of age on survival. Resistance to desiccation was related to the growth phase by drying cells harvested after 6 hr, 24 hr, 7 days, 33 days, and 120 days of incubation at 36 C. The relationship between resistance and cell age is shown in Table 2. Cells from 6-hr cultures exhibited the greatest susceptibility. Even with rapid desiccation under vacuum, only one viable organism was recovered from glass squares assayed immediately after the drying process.

The 24-hr cultures yielded cells with in-

TABLE 1. *Survival after drying of P. aeruginosa cells deposited on glass squares*

Time after drying	Mean no. of cells surviving on glass squares		
	Trial I	Trial II	Trial III
0	350	518	1,250
20 min	0	52	16
40 min	6	16	10
60 min	4	14	2
120 min	0	22	2
1 day	0	0	0
2 days	0	0	0
3 days	0	0	0
4 days	0	0	0

TABLE 2. *Survival of P. aeruginosa cells harvested from different age cultures desiccated on glass squares and held at 25 C and 53% RH*

Age of culture (days)	Mean no. of cells surviving on glass squares				
	Immediately before drying	Immediately after drying	Hr at 25 C and 53% RH		
			24	48	72
1/4	254	1	0	0	0
	846	0	0	0	0
	86,200	0	0	0	0
1	358	313	0	0	0
	584	376	0	0	0
	975	314	0	0	0
7	810	495	5	0	1
	1,305	821	13	1	0
	2,271	1,561	56	29	2
33	298	112	0	0	0
120	509	159	0	0	0
	790	226	0	0	0
	7,059	2,130	1	0	0

creased resistance, as compared with organisms from 6-hr cultures. Viable cells were recovered immediately after the drying process; however, complete loss of viability occurred within 24 hr after desiccation.

Cells harvested from 7-day cultures exhibited the greatest resistance to adverse effects of desiccation. A comparatively large number survived the drying process. Viability decreased rapidly within the first 24 hr at 25 C and 53% RH; however, viable cells were recovered up to 72 hr after desiccation. No survivors were recovered from glass squares processed on the 4th to 6th day after desiccation.

The pattern of increasing resistance with increasing age observed for cells harvested at 6 hr, 24 hr, and 7 days was not continued with the 33- and 120-day-old cultures. Although a large proportion of cells survived desiccation, only a single viable organism from the 120-day cultures was recovered after 24 hr at 25 C and 53% RH.

Effect of suspending medium on survival. To determine whether the precipitous death of *P. aeruginosa* OSU 64 was due, in part, to the nature of the broth-suspending medium, 6-hr cells were dried in bovine serum. No cells survived the drying process. Loss of viability was similar to that observed for cells dried in broth.

Effect of slime on survival. With prolonged incubation, extracellular slime accumulated in liquid cultures of *P. aeruginosa* OSU 64. Since 7-day cultures exhibited greater resistance to desiccation, cells harvested from 6-hr cultures were dried in the highly viscous cell-free medium from 7-day cultures to determine the effect of slime on survival (Table 3). No survivors were recovered immediately after the drying process. The death rate was similar to that observed for cells dried in 6-hr broth which contained no detectable slime. Further evidence that difference in resistance was probably not due to any extrinsic protective mechanism was observed with the behavior of 7-day cells dried in the comparatively unaltered broth from 6-hr cultures. A large number of 7-day cells survived the drying process, and viable cells were recovered after 24 hr at 25 C and 53% RH. The accumulation of slime was not necessary for 7-day cells to survive the drying process and 24 hr of the holding period.

Effect of incubation temperatures on survival. To determine the effect of growth temperature on resistance, cells cultured for 24 to 72 hr at 20, 36, and 42 C were exposed to desiccation. A large proportion of the cells from cultures incubated at 36 and 42 C survived the drying process. However, for cells cultured at 20 C, a relatively small number were recovered immediately after drying, even though the number exposed initially was greater than that for cultures incubated at 36 or 42 C. No viable cells from any of the cultures were recovered from glass squares desiccated for 24 hr or longer.

Resistance of strains from natural habitats. The survival of desiccated organisms from 5 nonlaboratory strains was determined with cells harvested from 24-hr cultures. Viable cells were recovered immediately after the drying

TABLE 3. *Effect of slime in suspending medium on survival of desiccated P. aeruginosa*

Age of cells	Suspending medium	Mean no. of cells surviving on glass squares		
		Immediately before drying	Immediately after drying	After 24 hr at 25 C and 53% RH
6 hr	With slime	604	0	0
		912	0	0
7 day	Without slime	1,230	859	13

process from only two strains, one isolated from a wound and the other from a water faucet. There were no survivors after 24 hr at 25 C and 53% RH.

DISCUSSION

In general, the results indicate that *P. aeruginosa* is highly susceptible to desiccation. The data were accumulated with relatively small populations to ensure that extracellular conditions did not mask the susceptibility of individual cells. Deposition of large populations on the glass squares undoubtedly would have resulted in much longer survival periods, since large numbers usually contain a few exceptionally hardy cells which can better withstand the effects of deleterious agents than the overall population. With desiccation of suspensions containing large numbers of organisms, however, there is a likelihood that the physical state of some cells may influence survival. Organisms occluded in cellular agglomerations are protected against rapid and excessive dehydration. Recovery of a greater number of viable cells under such conditions does not necessarily reflect a cellular characteristic but rather the influence of extrinsic factors.

Cells harvested during the logarithmic growth phase from cultures incubated at 36 C were more susceptible to the effect of drying than those from older cultures. The high susceptibility of cells which were several hours old to drying has also been observed with *Escherichia coli* (4). Although a high proportion of the cells from 24-hr cultures survived the drying process, the failure to recover viable cells after only 24 hr at 25 C and 53% RH indicated that resistance to desiccation was low even with organisms from the early stationary growth phase. The hardiest cells were recovered after 7 days of incubation, when growth was in the decline phase. Extension of the incubation period to 33 and 120 days did not result in a concomitant increase in resistance. Survival of cells from these cultures was similar to that observed for 24-hr cells.

Although resistance of *P. aeruginosa* to the action of chemotherapeutic agents as well as increased pathogenicity has been attributed to the presence of extracellular slime (1, 5), protective action against the effects of desiccation was not observed in the present study. The loss of viability when 6-hr cells were dried in cell-free medium from 7-day cultures was similar to that for cells dried in 6-hr broth. The accumulation of slime in 7-day cultures was readily evident when the medium was transferred with a pipette or wire loop. The survival of 7-day cells for 24 hr after desiccation in 6-hr medium which contained no perceptible slime suggests that the increased resistance is a property of the organism rather than due to extrinsic factors. It may be that some slime material remained firmly adhered to the cell surface as a tenacious, discrete covering on the 7-day cells transferred to 6-hr broth. If this slime layer remained intact during the drying process, a protective action may have occurred. This could not be discerned from these experiments.

Examination of strains from natural sources did not reveal any unusual resistance to desiccation. Even though the organisms had been recently subjected to selective pressures in a natural habitat, survival of cells from 24-hr cultures was similar to that observed for *P. aeruginosa* OSU 64. The organisms were not better adapted to withstand the inimical conditions of desiccation. Under certain conditions, such as the occlusion of cells in a highly impervious substance, loss of moisture might be retarded and survival increased. However, without extracellular protection, *P. aeruginosa* cells are not likely to remain viable under natural conditions for prolonged periods.

Previous investigations have shown that *P. aeruginosa* can readily proliferate (3) as well as survive for months in distilled water (2). In a nutrient broth which was periodically replenished with sterile distilled water, the organism remained viable for over a year (9). Inanimate wet habitats have been implicated as sources of organisms for outbreaks of nosocomial infections. In view of the susceptibility of *P. aeruginosa* to desiccation, thorough drying of such sources would appear to be an effective means of preventing the spread of *P. aeruginosa*.

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