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Inoculum Density-Dependent Mortality and Colonization of the Phyllosphere by *Pseudomonas syringae*

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Pseudomonas syringae **inocula containing cell concentrations ranging from 10^5 to 10^9 cells per ml were applied to the primary leaves of bean plants. The plants were incubated under conditions of high temperature and illumination and low relative humidity. Bacterial mortality rates and the proportional population decline of the inoculum were lowest at the highest inoculum concentrations. Addition of a high concentration of heat-killed cells to the inoculum containing a low concentration of viable cells significantly reduced both the mortality rate and the proportional population decline of the viable cells. The mechanisms underlying this density-dependent mortality may include cooperative protective effects of extracellular factors, such as bacterial extracellular polysaccharides, and physical protection by neighboring cells. Although epiphytic populations derived from inoculum concentrations of 10^8 or 10^9 cells per ml tended toward 10^6 CFU/g, the presumed carrying capacity of the leaf, populations derived from lower inoculum concentrations never achieved this carrying capacity. Assuming that epiphytic populations of *P. syringae* reside in discrete protected sites, our results suggest that at low inoculum concentrations, following a period of environmental stress, the number of viable cells may have dropped to zero in some sites; hence, the carrying capacity of the leaf could not be achieved.**

The leaf surface is considered to be an inhospitable environment for microbial growth. Free moisture is frequently absent from the leaf surface, and leaves are subject to rapid fluctuations in relative humidity (3, 4). Bacteria are sensitive to desiccation, and although fluctuations in relative humidity are attenuated by the laminar boundary layer, epiphytic populations are directly affected by relative humidity (3, 4). Bacteria artificially inoculated onto leaf surfaces in the field, or in growth chambers under conditions simulating the field environment, usually undergo a rapid decline in population size (1, 10, 11, 21, 37, 39, 40, 43). While many bacterial species can colonize the leaf surface under conducive humid conditions, maintenance of a population under stressful or fluctuating environmental conditions appears to be limited to epiphytes (27).

Survival of epiphytic bacteria under adverse environmental conditions on the leaf surface may be achieved through either avoidance or tolerance of stress. Epiphytic bacteria may occupy sites which provide protection against desiccation stress, such as the depressions between cells, substomatal cavities, or the base of trichomes (22). Survival of bacterial inocula on leaves of pubescent species is generally higher than that on glabrous species (10, 27). Alternatively, epiphytic species may have developed mechanisms enabling them to tolerate environmental stress. There is evidence that bacteria on leaf surfaces are surrounded by a layer of extracellular polysaccharides (EPS) (24). The EPS matrix in which the cells are embedded may modify the physical and chemical environment experienced by the cells in a manner favorable to survival and growth (24). The stigmatic surface is frequently colonized by epiphytic species, even under conditions of low relative humidity (38). The presence of hygroscopic polysaccharides of host and bacterial origins has been hypothesized to be the reason for this (38). Such a matrix would be analogous to the biofilms produced by aquatic microbes that provide protection against predation and toxic chemicals (12).

While the persistence or survival of epiphytic phytopathogenic bacteria either throughout the season or on alternative hosts has received a great deal of attention (13, 32), the factors affecting survival of naturally generated or artificially cultured bacterial inocula on aerial plant surfaces have received much less attention. Some studies have addressed the effects of the physiological state (34, 35, 43), the effects of environmental conditions (22, 35, 36, 39, 40), and the effects of adjuvants (9). Recent interest in the environmental application of genetically engineered microorganisms has also prompted the study of the effects of aerosolization on bacterial survival (26, 42). An understanding of bacterial mortality on aerial plant surfaces is pertinent to attempts to model the survival and persistence of genetically engineered microorganisms in the environment and also to the application of bacterial biological control agents of foliar pests and diseases in situations in which the inoculated organism must survive and colonize the leaf.

We report here the density-dependent survival of bacterial cells applied to leaves, the influence of initial cell density on final population size, and possible models for bacterial colonization of leaves under stressful environmental conditions.

MATERIALS AND METHODS

Preparation of bacterial inocula and inoculation of plants. The origin and characteristics of *Pseudomonas syringae* MF714R have been described previously (43). *P. syringae* MF714R was cultured on King's medium B (KB) plates for 24 h at 28°C. Bacterial cells were scraped from the plates and suspended in potassium phosphate buffer (0.01 M, pH 7.0). The viable cell concentration in these suspensions was estimated turbidimetrically, and all suspensions were diluted to the appropriate concentration. Bacterial suspensions were sprayed to run off onto bean plants (*Phaseolus vulgaris* cv. Bush Blue Lake 274) at the primary leaf stage. Each treatment was

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applied to five replicate pots of beans. The pots of beans were randomized within a growth chamber which maintained high light intensity (ca. 100,000 microeinsteins/m^2), low relative humidity (45 to 50%), and a temperature of 26°C.

In order to test the possible protective effect of a high concentration of dead cells in the inoculum, a suspension of *P. syringae* MF714R was subjected to heat (60°C for 45 min). A small volume containing the appropriate number of viable cells was then added to the heat-killed cells, to achieve a final concentration of 10^7 viable cells per ml and approximately 10^9 dead cells per ml.

Enumeration of bacterial populations. Twenty individual leaves, four from each of five replicate pots, were harvested from each treatment at approximately 2-h intervals following inoculation. The leaves were immersed in 20 ml of washing buffer (0.1 M potassium phosphate buffer [pH 7.0] containing 0.1% Bacto Peptone [Difco]) and sonicated (Bransonic 8200) for 7.0 min. Appropriate 10-fold dilutions were plated on KB amended with 50 μg of benomyl per ml, 100 μg of cycloheximide per ml, and 100 μg of rifampin per ml. The mean log-transformed *P. syringae* population size estimated from 20 individual leaves was determined for each treatment and sampling time. The time required for one-half of the cells to die after inoculation, the half-life of viability of the inoculum, was estimated from the slope of the regression of $\log_2$ (population size) against time. Regression analysis was performed by using SAS (release 6.04; SAS Institute, Cary, N.C.). The proportional population decline that occurred during different time intervals after inoculation was calculated from the ratio of the arithmetic mean of log-transformed population sizes at the beginning and end of each interval. All population datum sets were analyzed for conformance to the lognormal distribution as described by Shapiro and Wilk (33).

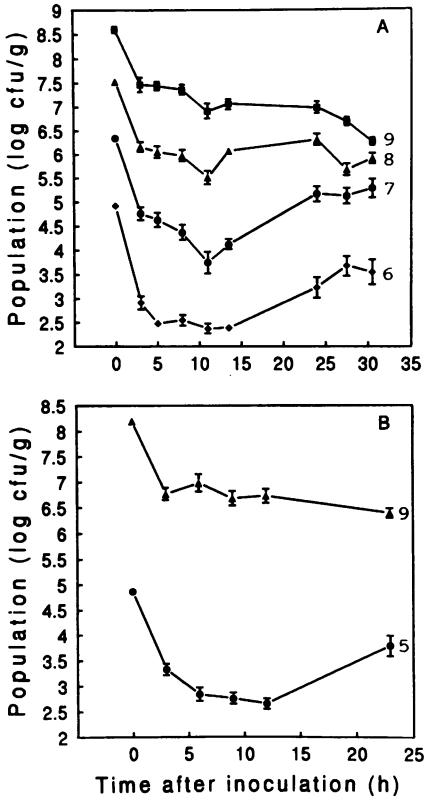

FIG. 1. Survival of *P. syringae* inocula containing various cell concentrations inoculated onto bean leaves and incubated under stressful environmental conditions. (A) 10^9 cells per ml (squares), 10^8 cells per ml (triangles), 10^7 cells per ml (circles), and 10^6 cells per ml (diamonds). (B) 10^9 cells per ml (triangles) and 10^5 cells per ml (circles).

RESULTS

In order to determine the effect of the inoculum concentration on subsequent survival of cells of *P. syringae* on leaves, suspensions with different concentrations were spray inoculated onto the primary leaves of bean plants. Immediately following inoculation, the plants were exposed to stressful environmental conditions. The number of viable cells from inocula applied at a range of concentrations decreased at different rates in the 12-h period following inoculation (Fig. 1). Within the first 4 h after inoculation, the half-life of viability of the cells was longest at the highest inoculum concentrations (Table 1). The proportional population decline in the first 4-h period and the subsequent 8-h period, determined from the ratio of the population sizes at the beginning and end of each period as well as the decline over the entire 12-h period, were consistently smallest at the highest inoculum concentrations (Table 1). After this 12-h period, the populations derived from the inoculum with the highest concentration continued to decrease at a very low rate, while the populations derived from the inocula with lower concentrations started to increase (Fig. 1).

The populations of strain MF714R on plants inoculated with either 10^9 or 10^8 cells per ml approached a population size of approximately 2×10^6 CFU/g (Fig. 1A). In contrast, the populations of strain MF714R on plants derived from lower inoculum concentrations appeared to stop increasing in size at less than 10^6 CFU/g (Fig. 1A). Extended incubations were necessary to determine whether the populations derived from low-concentration inocula would eventually reach the same size as those derived from high-concentration inocula. Inoculation of plants with high and low cell concentrations followed by extended incubation (up to 75 h) under stressful environmental conditions indicated that those populations derived from low numbers of applied cells were unable to attain the same population size as were high numbers of applied cells (Fig. 2).

The Shapiro-Wilk test (33) was used to determine the conformance of the distribution of the epiphytic populations on individual leaves at each sample time to lognormality. While the distributions of epiphytic population sizes on individual leaves at sample times immediately following or shortly after inoculation were described equally well by the normal or lognormal distribution, at later sample times the distributions of epiphytic population sizes on individual leaves were best described by the lognormal distribution. No differences were observed in the nature of the change in the distribution of epiphytic population sizes between high and low inoculum concentrations.

To investigate the factors contributing to the lower mortality rate of bacteria on plants inoculated with high numbers of cells, a low concentration of viable cells of strain MF714R was added to a high concentration of heat-killed cells. The death curve observed for the viable cells plus heat-killed cells was more typical of that following inoculation with a high concentration of viable cells (Fig. 3). Similar, though less-pronounced, protective effects also were observed in two other experiments. In the 4-h period after inoculation of plants, the half-life of viability of the inoculum containing 10^7 viable cells per ml and 10^9 dead cells per ml was similar to the half-life of viability of

TABLE 1. Death of *P. syringae* inocula on leaf surfaces under stressful environmental conditions

Expt[a]	No. of cells applied/ml	0–4 h postinoculation		4–12 h postinoculation		Total population decline (t_0/t_4)
		Half-life[b] (min)	Population decline[c] (t_0/t_1)	Half-life (min)	Population decline (t_1/t_4)	
1A	10^9	47.9	13.6	221.7	3.5	47.2
	10^8	39.4	23.7	206.0	4.5	106.7
	10^7	34.3	37.8	134.8	10.4	395.3
	10^6	26.9	103.0			358.14
1B	10^9	38.1	26.6	212.2	1.1	29.0
	10^5	35.2	34.3	214.2	4.7	160.6
2A	10^8	25.2	27.2	77.6	4.6	123.8
	10^6	15.9	188.0	71.1	5.6	1,051.0
3	10^9	44.4	6.5	111.7	25.4	165.9
	10^7	13.6	447.4	109.9	17.0	7,630.0
	$10^7 + 10^9$ (dead)	45.8	6.2	252.5	1.6	9.8

[a] Experiment numbers correspond to the respective figures.
[b] The inoculum half-life (in minutes) determined from the slope of the regression of $\log_2$ (population) size against time.
[c] The proportional population decline of the inoculum determined from the ratio of the arithmetic mean log-transformed population sizes at the beginning (t_0) and end of each time period (t_1 and t_4, 4 and 12 h postinoculation, respectively).

the inoculum containing 10^9 viable cells per ml and much longer than the half-life of viability of the inoculum containing only 10^7 viable cells per ml (Table 1). The decrease in viable population size of strain MF714R with an inoculum containing 10^7 viable cells per ml and 10^9 dead cells per ml or 10^9 viable cells per ml was approximately 6-fold, compared with approximately 450-fold with an inoculum containing 10^7 viable cells per ml (Table 1). While decreases in cell viability were slower after 4 h for both treatments, the half-life of viability was longest, and the proportional population decline was smallest when 10^7 viable cells per ml and 10^9 dead cells per ml were applied together (Table 1). The total population decline was much less for the inoculum containing 10^7 viable cells per ml and 10^9 dead cells per ml than for the inoculum containing only 10^7 viable cells per ml (Table 1).

DISCUSSION

When a *P. syringae* inoculum was applied to leaf surfaces and the plants were incubated under stressful environmental conditions in a growth chamber, cell concentration affected both the bacterial mortality rate (the half-life of viability of the inoculum) and the proportional population decline. The rate of decrease of viable population size was generally inversely proportional to the logarithm of the numbers of bacteria that had been applied to the plant surface. The presence of a high concentration of heat-killed cells reduced the mortality rate and the proportional population decline of an inoculum containing a low concentration of viable cells, compared with unamended inocula containing the same low concentration of viable cells. While part of the protective effect afforded by the presence of a high concentration of heat-killed cells may have been due to substances released by the lysis of some of the cells, the reduced mortality of the viable cells indicates that protection at high cell concentrations resulted from a mechanism not requiring metabolic activity. The results of this study suggest either that the bacterial cells at high concentrations formed agglomerations on the leaf surface in which the physical presence of other cells provided protection against environmental stress or that there was some cooperative protective effect conferred by cell products from adjacent cells which was not afforded to more widely dispersed cells.

The relative contributions to epiphytic survival made by physical protection through agglomeration or cooperative protective effects from cell products are hard to assess. Assuming the inoculated bacterial cells to be dispersed evenly over the leaf surface, on average, cells would not be closely packed at the inoculum concentrations used here. However, some agglomeration of inoculated cells undoubtedly occurred as cells are frequently found preferentially in depressions on the most hydrophilic areas of the leaf surface. Hence, some protection was probably provided by physical occlusion or shielding of cells from the physical environmental conditions. Nevertheless, it appears likely that cooperative effects due to the presence of EPS, or other extracellular products, play an important role in the superior survival of cells at high inoculum concentrations. The desiccation tolerance of bacteria, including *Pseudomonas aeruginosa* (30) and *Erwinia amylovora* (25), on artificial surfaces has been found to be increased by the presence of EPS, the desiccation tolerance of *Pseudomonas* spp. in soil has also been correlated with EPS production (31), and mucoid strains of *Escherichia coli*, *Erwinia stewartii*, and *Acinetobacter calcoaceticus* were more desiccation resistant than isogenic nonmucoid mutant derivatives (28). Further, density-dependent mortality was observed with an EPS-overproducing mutant of *P. syringae* B728a but not with the parental strain (1b). The latter observation suggests not only that the density-dependent mortality may be strain specific but also that the observed superior survival at high inoculum concentrations may be primarily due to cooperative protective effects of bacterial EPS.

The phenomenon of density-dependent mortality observed here could be one factor contributing to the skewed or lognormal distribution observed for epiphytic bacterial populations on subsections of individual leaves (17). Leaves having a normal distribution of populations in colonization sites due to uniform rates of immigration of cells to a leaf (or inoculation) would exhibit a progressively more-left-hand-skewed population distribution with density-dependent population decline. Sites which had a high population density would be subject to a lower mortality rate and proportional population decline than would sites containing a low population density. Density-dependent mortality may also contribute to the skewed or lognormal distribution of epiphytic population sizes

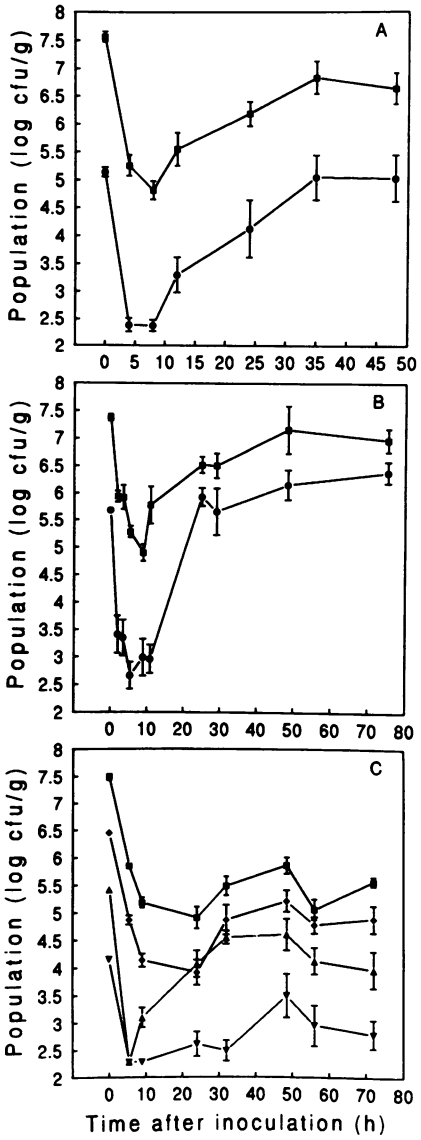

FIG. 2. Survival of *P. syringae* inocula containing various cell concentrations inoculated onto bean leaves and incubated under stressful environmental conditions. (A) 10^8 cells per ml (squares), 10^6 cells per ml (circles); (B) 10^8 cells per ml (squares) and 10^6 cells per ml (circles); (C) 10^8 cells per ml (squares), 10^7 cells per ml (diamonds), 10^6 cells per ml (triangles), and 5×10^4 cells per ml (inverted triangles).

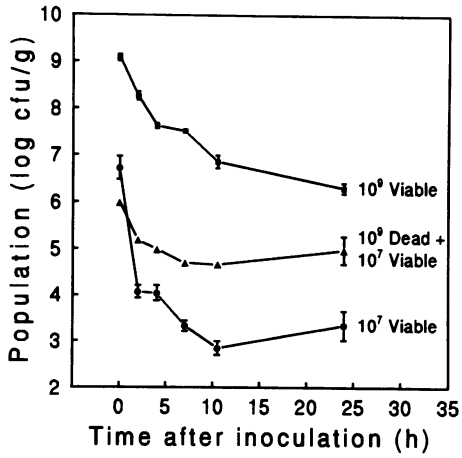

FIG. 3. Survival of *P. syringae* inocula containing high and low cell concentrations or a high concentration of heat-killed cells amended with a low concentration of viable cells inoculated onto bean leaves and incubated under stressful environmental conditions.

observed on individual leaves (14, 15). Leaves supporting high population densities would be subject to higher mortality rates than would those supporting low population densities. The progression from a normal to a lognormal distribution of epiphytic population sizes on individual leaves following inoculation and subsequent death of the inoculum in these experiments supports this concept. While the lognormal distribution of epiphytic populations on individual leaves has been previously attributed to differential growth rates (6), it seems that a combination of differential growth rates and mortality rates may be involved.

The assessment of risk associated with the environmental application of genetically engineered microorganisms has relied on mortality data from microcosms to assess the survival of recombinant bacteria on plant surfaces (1, 29) and computer models using these data to estimate the survival and dispersal of bacteria applied to the foliage of crop plants in the field (19, 20). It is clear that the accuracy of predictions by such models is dependent on accurate estimates of mortality rates in the natural environment; however, such data are scarce (23), and even the factors affecting microbial mortality rates are incompletely understood. Bacterial mortality rates are affected by several factors, including environmental conditions (22, 35, 36, 39, 40) and the physiological state of the cells (34, 35, 41, 43). While the experiments in this study were conducted under controlled conditions in a growth chamber, essentially a microcosm, they do suggest that cell density is yet another factor which may affect mortality rates under field conditions. Hence, inoculum cell densities should also be considered in future models predicting the survival and dispersal of genetically engineered microorganisms.

The epiphytic populations derived from the highest inoculum concentrations used in these experiments tended toward a final size which can be considered the carrying capacity of the leaf surface under this set of environmental conditions. This strongly suggests that resource availability limits the bacterial population size on leaves. While applications of the highest inoculum concentrations resulted in the lowest mortality rates, these population sizes exceeded the carrying capacity of the leaf and the population size continued to decline. Presumably, the population size declines until the available resources in the protected sites match the resource demands for the maintenance of the cells surviving in those sites. While slightly lower inoculum concentrations were subject to a higher mortality rate, leading to population sizes transiently below the carrying capacity of the leaf, multiplication of cells surviving in protected sites on the leaf surface permitted the increase of these populations up to the carrying capacity. The observation that population sizes converge in this way lends strong support to the concept of the carrying capacity as an equilibrium between resource availability and resource use in the protected sites. Similar equilibration of bacterial population sizes has been observed following inoculation of high and low numbers of cells into soils (5, 30), the spermosphere (7), and the rhizosphere (2). In contrast to the high inoculum concentrations, populations derived from the lowest inoculum concentrations did not reach the carrying capacity of the leaf, even following

extended incubation. When relatively low numbers of cells were applied to leaves, either some sites remained uninoculated or the populations in some sites dropped to zero following imposition of stressful environmental conditions. While *P. syringae* is motile on the leaf surface (11), motility cannot occur in the absence of free water; hence, recruitment of cells from neighboring sites would not be possible under these conditions and some sites would remain uncolonized. Therefore, the carrying capacity of the leaf would not be achieved. Similar results have been observed with other epiphytic phytopathogenic *P. syringae* strains (1a). Analogous behavior occurs when phytopathogenic bacteria are vacuum infiltrated into leaves of compatible host plants (8, 44) or are inoculated into the rhizosphere (16, 18, 42). Following inoculation of these plant surfaces, final bacterial population sizes are proportional to the initial cell concentration, even after extended incubation. It is probable that in all these cases, the bacterial cells are restricted to discrete sites which are not fully exploited when only a few cells are inoculated and redistribution of cells to uncolonized sites is restricted by the absence of free water.

The results obtained here and in the study by Wilson and Lindow (43) suggest that epiphytic bacterial populations that drop below 10^4 to 10^5 CFU/g on dry leaves are unable to recover to the carrying capacity. This suggests that the primary leaf of a bean plant provides between 10^4 and 10^5 protected sites for *P. syringae*, assuming that the lowest population size which can recover to the carrying capacity occurs when there is on average one cell per protected site. The location of the cells surviving in the phyllosphere and the nature and distribution of the protected sites deserve further investigation.

Biological control of foliar pathogens frequently fails because of poor survival of applied inocula. While the application of lyophilized cells (21), older cells (34, 35), or cells grown under conditions that simulate the leaf surface (43) may apparently improve survival in the short term, long-term survival is dependent upon colonization of all the protected sites in the phyllosphere. These data suggest that optimal colonization of foliar surfaces in the field, when dispersal of bacteria across the leaf is restricted, would be achieved by the use of inocula consisting of a cell concentration sufficiently high that, after the anticipated cell death, at least one cell per protected site would remain. This would enable the biological control agent to attain the carrying capacity of the leaf.

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