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Relationship of Total Viable and Culturable Cells in Epiphytic Populations of *Pseudomonas syringae*

M. WILSON* AND S. E. LINDOW

Department of Plant Pathology, 147 Hilgard Hall, University of California, Berkeley, California 94720

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The direct viable count method, used to detect viable but nonculturable bacteria in aquatic systems, was modified to examine epiphytic populations of *Pseudomonas syringae*. Viable-population sizes determined from the number of cells that elongated when incubated with yeast extract and nalidixic acid were compared with those determined by the conventional plate count method. The plate count method accurately determined the number of viable cells in epiphytic *P. syringae* populations in a state of active growth under conditions of high relative humidity. The plate count method also accurately determined the number of viable cells in *P. syringae* inoculum, or a growing *P. syringae* population, subject to desiccation stress under conditions of low relative humidity. In epiphytic populations of *P. syringae* older than 80 h, however, the plate count underestimated the viable-population size by about two- to fourfold, suggesting that up to 75% of the *P. syringae* population was nonculturable. These nonculturable cells may have entered a starvation-survival state, induced by low nutrient availability in the phyllosphere environment. Epiphytic *P. syringae* populations undergoing rapid size changes due to growth and death under fluctuating environmental conditions in the field should be accurately enumerated by the plate count method. However, the possible underestimation of viable-population size under some circumstances should be considered in epidemiological studies of phytopathogenic bacteria and when genetically engineered microorganisms in terrestrial ecosystems are monitored.

The accuracy of the plate count method which is used routinely for the enumeration of viable bacterial populations in natural environments is limited by the culturability of the target population. It has been known for some time that bacterial cells subject to abiotic environmental stress and those exposed to sublethal concentrations of antimicrobial agents may be physiologically debilitated to such an extent that they are unable to grow on media containing selective agents (30). More recently, it was demonstrated that when the enteric bacterial pathogens *Streptococcus faecalis* and *Escherichia coli* were exposed to certain aquatic environments, the cells became physiologically injured and were not culturable on selective media (4). In the marine environment, large differences between bacterial numbers determined by the plate count method and those determined by direct microscopic methods have been observed (25). Direct microscopic methods, however, are unable to distinguish between living and dead cells and may overestimate numbers of viable bacteria (12). Kogure et al. (26) developed a microscopic method in which viable cells in marine environments can be distinguished and enumerated. In this direct viable count method, a water sample is amended with yeast extract (0.025%) and nalidixic acid (0.002%). While the yeast extract provides nutrients for cell growth, the nalidixic acid inhibits DNA synthesis (19), preventing cell division; hence, substrate-responsive (viable) cells elongate. The elongated cells are then stained with a DNA-specific fluorochrome such as acridine orange (3, 9, 16) or 4′,6-diamidino-2-phenylindole (DAPI) (9, 22, 33), counted by concentration onto polycarbonate filters (21), and then examined by epifluorescence microscopy (9).

Application of the direct viable count method has shown that when they are exposed to oligotrophic aquatic environments, several bacterial pathogens of humans and animals exhibit a viable but nonculturable phase, in which the direct viable count of the population is higher than the plate count. These pathogens include *Aeromonas salmonicida* (2), *Campylobacter jejuni* (34), *E. coli* (41), *Legionella pneumophila* (23), *Salmonella enteritidis* (36), *Vibrio cholerae* (5, 11, 41), and *Vibrio vulnificus* (28, 32). These species apparently enter a nonculturable starvation-survival state when exposed to the oligotrophic environment (35). The technique has also been used to enumerate populations of *E. coli* injured by exposure to chlorine (39). While the relationship between nonculturable and injured cells remains unclear, both types of cells are undetectable by the conventional plate count method (37).

The viable-but-nonculturable-phase phenomenon has been recognized as a problem in the detection and monitoring of genetically engineered microorganisms (GEMs) which have entered lakes or rivers (11, 12, 40). It is not known, however, whether the viable-but-nonculturable-phase phenomenon occurs in terrestrial-habitat bacterial populations, such as epiphytic bacteria. Studies on the epidemiology of phytopathogenic bacteria rely heavily on the plate count method for population monitoring, and the existence of large populations of either viable but nonculturable cells or physiologically injured cells would pose serious questions about the validity of such studies. We are aware, however, of only one published study in which any attempt was made to compare the population size of an epiphytic bacterial species determined by plate counts with the population size determined by direct microscopic observation (15). In that study, there was a correlation between total counts of rod-shaped cells, similar in size to *Pseudomonas syringae*, on bean leaves and viable counts of *P. syringae* from washings of comparable leaves. Apart from epidemiological studies, the occurrence of viable but nonculturable cells or of injured cells in epiphytic bacterial populations would also pose

* Corresponding author.

serious problems for the monitoring of GEMs in terrestrial ecosystems with the plate count method. This could be particularly problematic after mitigation procedures employing biocides which may result in the production of sublethally injured cells (14).

Epiphytic bacterial populations are subject to various environmental stresses, including UV irradiation (6, 24) and fluctuating relative humidity (7, 8). Further, epiphytic populations residing on crop plants may be exposed to bactericidal compounds such as copper hydroxide (29). Any of these stress factors may result in sublethal injury to a portion of the population. In addition to these abiotic stresses, an epiphytic bacterial population may be subject to conditions of low nutrient availability. *Pseudomonas* spp. have been shown to exhibit a viable but nonculturable phase upon exposure to an oligotrophic aquatic environment (10, 17, 40), and nutritional stress in the phyllosphere may result in some level of nonculturability. This study examined epiphytic *P. syringae* populations for injured cells produced by desiccation stress or viable but nonculturable cells produced by extended incubations resulting in conditions of nutrient depletion.

MATERIALS AND METHODS

Production of bean plants with low numbers of epiphytes. Bean seeds (*Phaseolus vulgaris* cv. Bush Blue Lake 274) were surface sterilized by a 30-s immersion in 70% ethanol and then a 2-min immersion in a 0.5% sodium hypochlorite solution. The seeds were washed twice in sterile distilled water before being planted in an autoclaved peat-sand mixture in clay pots. The pots and surface-sterilized seeds were placed in a surface-sterilized Plexiglas chamber into which filter-sterilized air was introduced to control the temperature and to produce positive pressure to prevent contamination. The seed pots were watered with sterile distilled water introduced into the chamber through sterile plastic tubing. Bean plants were grown until the primary leaves had fully expanded (about 10 to 14 days).

Inoculation and incubation of plants. Bean plants were removed from the sterile chamber and used immediately to minimize contamination from airborne microflora. A sample of 10 control leaves was removed prior to inoculation. *P. syringae* MF714R, cultured on King's medium B (KB) for 18 h at 25°C, was suspended in sterile potassium phosphate buffer (pH 7.0; 0.01 M), and the cell concentration was adjusted turbidimetrically to approximately 10^7/ml. The upper and lower surfaces of the bean leaves were sprayed with inoculum until runoff. The bean plants were subsequently incubated in three different ways: (i) the plants were placed in a growth chamber at 20°C and enclosed in plastic bags for the duration of the experiment to maintain a high relative humidity around the leaves; (ii) the plants were placed in a growth chamber at 20°C and enclosed in plastic bags for 48 h, and then they were placed in a growth chamber and incubated at 25°C and 45 to 50% relative humidity; (iii) the plants were placed directly in a growth chamber and incubated at 25°C and 45 to 50% relative humidity.

Sampling and processing of leaves. Ten leaves were sampled at appropriate intervals during incubation. Each leaf was placed in a tube containing approximately 20 ml of sterile phosphate buffer and sonicated for 7 min in an ultrasonic cleaning bath (31). The leaf sonicate was divided into two portions for enumeration of the viable bacterial population; the plate count method was used for one portion, and the direct viable count method was used for the other.

Determination of the viable bacterial population size by the plate count method. Leaf sonicates were serially diluted in sterile phosphate buffer. Appropriate dilutions were plated on KB amended with 100 μg of rifampin per ml and 50 μg each of cycloheximide and benomyl (Benlate [Dupont Chemical Co.]) per ml. Colonies were counted after 48 to 60 h of incubation at 25°C. The population size of viable bacteria on each leaf was normalized for the weight of the leaf. The mean viable bacterial population size was derived from the $\log_{10}$-transformed population of 10 replicate leaves. The sonicates from uninoculated control leaves were plated on KB amended with 50 μg each of cycloheximide and benomyl per ml in order to determine the size of the resident bacterial population contaminating the leaves.

Determination of the viable bacterial population by the direct viable count method. A 10-ml sample of leaf sonicate was amended with 10 ml of incubation solution. The final mixture of sonicate and incubation solution contained 0.25% yeast extract, 8 or 10 μg of nalidixic acid per ml, and 50 μg of rifampin per ml. Some samples were also incubated in the absence of rifampin. The mixture was incubated for 24 h at 28°C on a rotary shaker at 100 rpm and was then fixed with 2% formaldehyde (13). At each time interval, a preincubation (control) sample that was not incubated with yeast extract and nalidixic acid was fixed with 2% formaldehyde. The entire sample or 10 ml of an appropriate dilution was then filtered through a 25-mm-diameter 0.2-μm-pore-size black polycarbonate filter (Poretics Corporation, Livermore, Calif.). The filters were washed by passing 10 ml of filtered sodium phosphate buffer (pH 8.0; 0.1 M) through the filter to remove excess formaldehyde.

Individual filters were stained by being placed on a 0.3-ml drop of a 1-μg/ml solution of DAPI for 2 min in the dark. The filters were washed twice by being placed on a 0.3-ml drop of sodium phosphate buffer for 5 min in the dark. Each filter was placed on a glass microscope slide, and a single drop of antifade (18) was placed on the filter, which was then covered with a glass coverslip.

The polycarbonate filters were examined by epifluorescence microscopy using a Nikon Optiphot microscope equipped with a UV 1-A excitation filter. The polycarbonate filter from the preincubation (control) sample was examined to determine the mean cell length of the bacteria on the leaf at that time. For each filter, the number of elongated bacterial cells, i.e., the number of cells longer than the mean cell length for the control filter (38, 39) was counted in 10 fields of view at a magnification of ×40. The mean number of elongated cells per field of view was converted to the estimated number of cells per filter by using a multiplication factor derived by division of the usable filter area by the field of view. The estimated number of cells per filter was then converted to the number of viable cells per gram of leaf tissue. The mean viable bacterial population was derived from the $\log_{10}$-transformed population of 10 replicate leaves.

Comparison of viable counts determined by the plate count method and direct viable count method. The mean viable bacterial populations per gram of leaf tissue at each sample time determined by the plate count method and the direct viable count method were compared by using the *t* test to determine whether statistically significant differences occurred.

RESULTS

Modifications of the direct viable count method. In these experiments, plants having extremely low levels of contam-

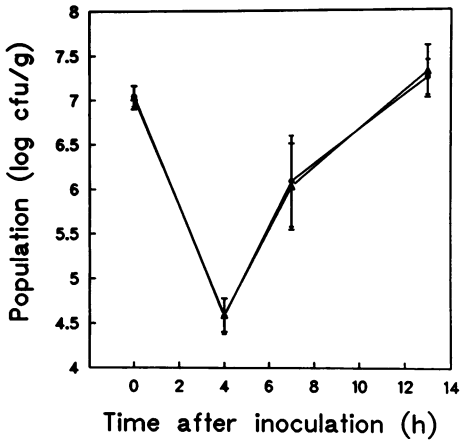

FIG. 1. Estimation of the *P. syringae* population size by plate counts on KB (triangles) and KB amended with 100 μg of rifampin per ml (circles) at various times after inoculum was applied to bean leaves, which then were incubated at a low (45 to 50%) relative humidity. Vertical bars, one standard error of the mean.

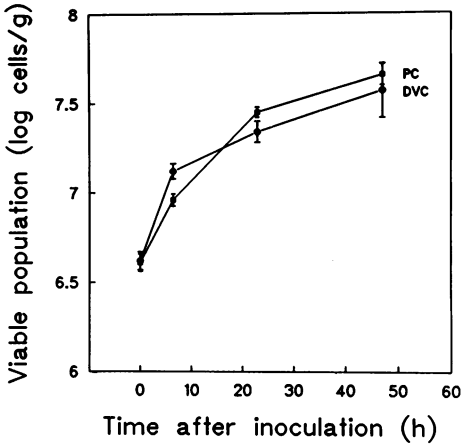

FIG. 2. Estimation of the viable *P. syringae* population size by the direct viable count (DVC) method and the plate count (PC) method at various times after inoculum was applied to bean leaves, which then were incubated at a high relative humidity. Vertical bars, one standard error of the mean.

inating epiphytes were required, because DAPI, the stain used in the direct viable count method, is not specific for the test organism *P. syringae*. The isolation method used to grow the bean plants was very effective at reducing the background levels of contaminating epiphytes, which on the majority of leaves were below the plate count detection limit (330 CFU per leaf). Although this level of contamination was extremely low, when plants were incubated at a high relative humidity for long periods, contaminants sometimes multiplied to high levels. Further, some of the contaminants were nalidixic acid resistant and multiplied to high numbers during the incubation step in the direct viable count method. The use of a rifampin-resistant *P. syringae* strain and incorporation of rifampin into the incubation mixture prevented multiplication of nalidixic acid-resistant contaminants and inhibited the elongation of nalidixic acid-sensitive contaminants while permitting the elongation of viable *P. syringae* cells. Incorporation of rifampin at 50 μg/ml into the incubation mixture had no significant effect ($P = 0.05$) on enumeration of viable cells. In one experiment, the viable-population sizes determined with and without rifampin amendment were 6.33 (standard error, 0.087) and 6.25 (standard error, 0.040), respectively. Incorporation of 100 μg of rifampin per ml into the enumeration medium used for plate counts did not significantly affect recovery of cells stressed by desiccation (Fig. 1).

The MIC of nalidixic acid for *P. syringae* MF714R was determined to be approximately 4 μg/ml. By the direct viable count method, optimal cell elongation was obtained at nalidixic acid concentrations higher than the MIC. The optimal nalidixic acid concentration for cells growing on leaves at high relative humidity was 10 μg/ml, and that for cells stressed by desiccation was 8 μg/ml. Cell elongation was at least partially inhibited at higher concentrations. The optimal incubation period was determined to be 24 h, because no further increases in numbers of elongated cells occurred after this time.

Comparison of viable counts determined by the plate count method and the direct viable count method. The culturability of *P. syringae* cells growing actively on plants under conducive conditions was examined. Inoculated plants were incubated at a high relative humidity in plastic bags and sequentially sampled for 60 h. The population size of *P. syringae* MF714R increased rapidly during the first 24 to 36 h and then started to level out (Fig. 2). There were no significant differences between the plate count and the direct viable count at any sample time between 0 and 60 h after inoculation in any of three replicate experiments (Fig. 2).

The culturability of *P. syringae* cells which had been growing on bean plants for an extended period was also examined. Inoculated plants were incubated at a high relative humidity in plastic bags and were sequentially sampled from 48 to 144 h. There were small but significant differences between the plate count and the direct viable count in two replicate experiments in the period between 84 and 144 h after inoculation (Fig. 3). Although estimates of viable-population sizes determined by the plate count and direct viable count methods differed by only two- to fourfold, this may suggest that between 50 and 75% of cells in the

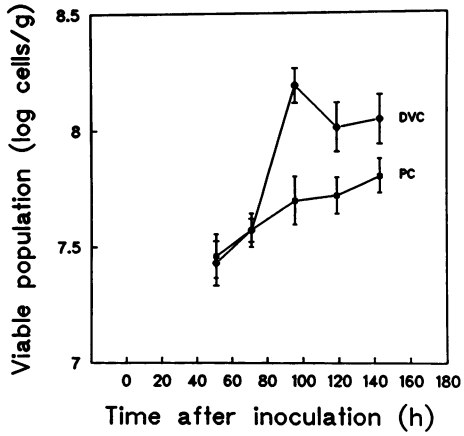

FIG. 3. Estimation of the viable *P. syringae* population size using the direct viable count (DVC) method and the plate count (PC) method at various times after inoculum was applied to bean leaves, which then were incubated at a high relative humidity for prolonged periods. Vertical bars, one standard error of the mean.

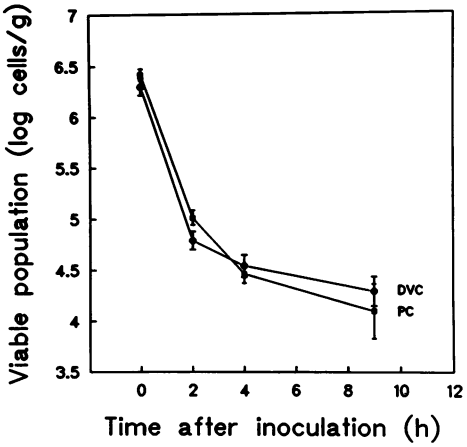

FIG. 4. Estimation of the viable *P. syringae* population size by the direct viable count (DVC) method and the plate count (PC) method at various times after inoculum was applied to bean leaves, which then were immediately exposed to low (45 to 50%) relative humidity. Vertical bars, one standard error of the mean.

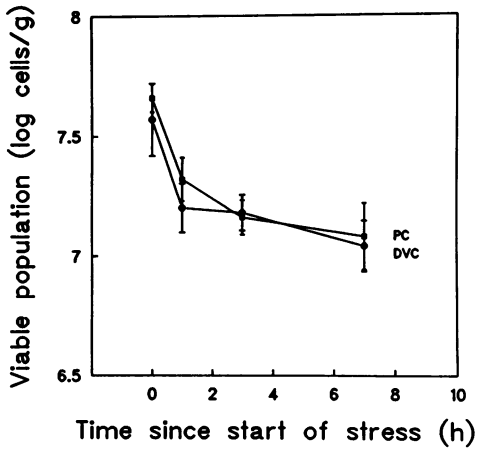

FIG. 5. Estimation of the viable *P. syringae* population size by the direct viable count (DVC) method and the plate count (PC) method. Inoculum was applied to bean plants which were incubated at high relative humidity for 48 h to establish large populations of *P. syringae*. Plants were then incubated at a low (45 to 50%) relative humidity, and samples were taken at the times after this change in relative humidity shown on the abscissa. Vertical bars, one standard error of the mean.

long-established population of *P. syringae* were viable but nonculturable.

To examine the effects of desiccation stress on recently applied inoculum, inoculated plants were placed directly in a growth chamber and incubated at 25°C and 45 to 50% relative humidity. The plate count and direct viable count population sizes of *P. syringae* decreased rapidly during the first 10 h after application of the stress (Fig. 4). There were no significant differences between the plate count and the direct viable count at any sample time in three replicate experiments (Fig. 4).

The culturability of *P. syringae* cells which had established populations on leaves but then were exposed to sudden desiccation stress was examined. Inoculated plants were incubated at a high relative humidity in plastic bags for 48 h in order to allow the *P. syringae* population to reach carrying capacity. The plants were then placed in a growth chamber and incubated at 25°C and 45 to 50% relative humidity. The plate count and direct viable count population sizes of *P. syringae* decreased slowly during the first 8 h after application of the stress (Fig. 5). There were no significant differences between the plate count and the direct viable count at any sample time in either of two replicate experiments (Fig. 5).

DISCUSSION

The direct viable count method of Kogure et al. (26) was successfully modified to examine epiphytic bacterial populations, enabling a comparison with the conventional plate count method which is routinely used for the enumeration of these populations. While the majority of studies of nonculturable cells have been conducted in sterile microcosms (2, 10, 23, 28, 32, 36), nonsterile environmental samples have been examined previously by specific immunofluorescent staining of elongated cells of the test organism (5, 22, 41). In this study, immunofluorescence was not used to detect *P. syringae* cells since this procedure could lead to underestimation of viable-population size if some cells are inadequately labelled. Instead, the nonspecific stain DAPI (9, 22, 33) was used and specificity was provided by the use of a rifampin-resistant *P. syringae* strain and incorporation of rifampin into the incubation mixture. Rifampin had no effect on enumeration of viable *P. syringae* cells by the direct viable count method. Further, the use of rifampin as a selective agent in the medium used for plate counts had no effect on the enumeration of stressed cells or of starved cells (17).

The method of Kogure et al. (26) was additionally modified by optimizing the nalidixic acid concentration. The optimal nalidixic acid concentrations for cell elongation were lower than the 20-μg/ml concentration used by Kogure et al. (26) and others (1, 5, 10, 23, 41). Other workers (38, 39) have also found it necessary to use lower nalidixic acid concentrations for optimal elongation of environmentally injured cells. The optimal concentrations were 8 μg/ml for cells stressed by desiccation and 10 μg/ml for cells growing under conducive conditions. Higher concentrations actually inhibited cell elongation or produced elongated cells which stained poorly, probably because of their low DNA content.

Viable-population sizes determined by the direct viable count method depend somewhat on the subjective determination of what represents an elongated cell. One objective approach, in which the number of cells longer than an arbitrary critical length in both the preincubation and the postincubation samples is recorded and the former subtracted from the latter to obtain the number of viable cells, was suggested by Al-Hadithi and Goulder (1). In our study, a less time-consuming approach, in which cells in the postincubation sample longer than the mean cell length for the preincubation sample were considered elongated (38, 39), was used. This approach appeared to be satisfactory, because for a growing *P. syringae* population, the direct viable count was indistinguishable from the plate count.

The modified direct viable count method employed in this study indicated that the plate count method accurately enumerated the viable bacterial population when that population was in a state of active growth. This suggests that none of the *P. syringae* cells growing under conducive conditions had entered the viable but nonculturable state. The plate count method also accurately enumerated the viable popu-

lation when epiphytic bacteria growing on leaves, or recently applied inoculum, were stressed by exposure to a low relative humidity. These data indicate that desiccation of *P. syringae* in the phyllosphere does not produce sublethally injured cells which, although viable, are not culturable on the media routinely used for enumeration of pseudomonads. Further, these data suggest that the plate count method can be used with confidence to enumerate viable populations when bacterial inoculum is applied in the field or when the epiphytic bacterial population is in a state of growth and death due to fluctuating environmental conditions. Such a situation probably exists at most times on leaf surfaces under field conditions, since large and rapid temporal changes in epiphytic-population sizes have been observed (20).

The plate count method did not accurately enumerate the viable population of *P. syringae* when the cells had been growing epiphytically under constant environmental conditions for more than 80 h. In *P. syringae* populations older than 80 h, the direct viable count was two- to fourfold higher than the plate count, indicating that up to 75% of the population may be nonculturable. This loss of culturability may have resulted from the depletion of nutrients in the phyllosphere habitats colonized by *P. syringae*. If the population was experiencing reduced metabolic activity and loss of culturability due to low nutrient availability, the situation may be analogous to the starvation-survival state observed for *Pseudomonas* spp. in aquatic systems (10, 17, 40). This finding is particularly relevant to the monitoring and detection of GEMs in terrestrial ecosystems, because if part of an epiphytic population has become metabolically inactive, or quiescent (27), the viable bacterial population size will be underestimated by the plate count method. The plate count may indicate that a GEM has disappeared from the ecosystem, whereas actually, the GEM is still present in low numbers in a viable but nonculturable, quiescent state.

The magnitude of the difference between the direct viable count and the plate count seen here for *P. syringae* cells is, however, much less than that observed for other species which exhibit the phenomenon. For example, differences of 10- to 100-fold have been observed with *E. coli* in oligotrophic aquatic environments (12, 41), and in *Vibrio* species, the plate count may indicate that the population has dropped to undetectable levels while the direct viable count still indicates viable populations of 10^4 to 10^5 CFU/ml (28, 32). While the effects of a two- to fourfold underestimation of *P. syringae* populations, which occurs only under limited circumstances, probably would pose little problem in estimates of population sizes in most epidemiological studies, higher levels of nonculturability may be observed with other epiphytic bacterial species or on different host plant species.

While this study has determined that desiccation stress of epiphytic *P. syringae* populations does not produce sublethally injured cells, it would be useful in the future to determine whether other environmental stress factors to which epiphytic populations are regularly exposed, such as UV irradiation (6, 24) and exposure to copper bactericides (29), do result in the production of such cells, which are not culturable on selective media. Additionally, although these results suggest that the number of viable but nonculturable cells of *P. syringae* is probably low under the fluctuating environmental conditions found in the field, direct assessment of nonculturable-population sizes in epiphytic bacteria on a range of host plants under field conditions would be useful.

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