Resident Bacteria on Leaves Enhance Survival of Immigrant Cells of *Salmonella enterica*

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ABSTRACT

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Although *Salmonella enterica* apparently has comparatively low epiphytic fitness on plants, external factors that would influence its ability to survive on plants after contamination would be of significance in the epidemiology of human diseases caused by this human pathogen. Viable population sizes of *S. enterica* applied to plants preinoculated with *Pseudomonas syringae* or either of two *Erwinia herbicola* strains was ≥10-fold higher than that on control plants that were not precolonized by such indigenous bacteria when assessed 24 to 72 h after the imposition of desiccation stress. The protective effect of *P. fluorescens*, which exhibited antibiosis toward *S. enterica* in vitro, was only ≈50% that conferred by other bacterial strains. Although *S. enterica* could produce small cellular aggregates after incubation on wet leaves for several days, and the cells in such aggregates were less susceptible to death upon acute dehydration than solitary cells (as determined by propidium iodide staining), most *Salmonella* cells were found as isolated cells when it was applied to leaves previously colonized by other bacterial species. The proportion of solitary cells of *S. enterica* coincident with aggregates of cells of preexisting epiphytic species that subsequently were judged as nonviable by viability staining on dry leaves was as much as 10-fold less than those that had landed on uncolonized portions of the leaf. Thus, survival of immigrant cells of *S. enterica* on plants appears to be strongly context dependent, and the presence of common epiphytic bacteria on plants can protect such immigrants from at least one key stress (i.e., desiccation) encountered on leaf surfaces.

The occurrence of human pathogenic bacteria on fresh fruit and vegetables has been associated with an increase in outbreaks of human disease in several industrialized countries. Although outbreaks of foodborne illnesses caused by *Escherichia coli* O157:H7 have received perhaps the most attention and cause the most loss of life, the incidence of illness due to contamination of a variety of edible plants by various strains of *Salmonella enterica* is also substantial (3,10,13,15,37,38). Non-typhoid salmonellosis caused by *S. enterica* is the most common bacterial foodborne illness in the United States (1). Although outbreaks of salmonellosis have historically been associated with consumption of meat and other animal products, uncooked fruit and vegetables are now considered among the most common sources of such contamination. Outbreaks associated with *S. enterica* contamination of cilantro, tomato, cantaloupe, and other crops appear to be increasing in frequency (3,10,13,15,37,38).

There is a growing appreciation that preharvest colonization of crops by *S. enterica*, rather than contamination that occurs post harvest, is associated with many cases of human disease. Surveys of both domestic and imported produce in the United States reveal that samples are commonly contaminated with *Salmonella* spp. (12,15,38). Despite the recognized environmental phase (45) and relatively high frequency at which this human pathogen is found on plants, little is known of its interactions with the plants on which it can establish (5,41). Despite the possibility that *Salmonella* spp. might establish sizable reservoirs on plants, animal hosts remain the probable source of these strains. *Salmonella* spp. have been demonstrated to colonize the aboveground parts of plants following application of manure (2,5,17), providing solid evidence that preharvest contamination of plants is a plausible route of contamination. Unfortunately, how pathogens such as *Salmonella* survive in the plant environment is very poorly studied (5,39,41).

The limited studies of the behavior of *Salmonella* spp. on plants suggest that, although they can multiply under favorable conditions such as relatively warm temperatures and in the presence of moisture, they are less fit than many other bacteria more commonly found on such plants (5,6). It is unclear how commonly such conducive conditions might be encountered on plants that simultaneously had been contaminated by *Salmonella* spp. *S. enterica* was unable to multiply on dry leaves, unlike certain other plant-associated bacteria such as *Pseudomonas syringae* (32). Factors that would influence its ability to survive after immigration to the plant are perhaps more significant in determining the persistence of epiphytic populations of *Salmonella* spp. This aspect of the epiphytic biology of *Salmonella* spp. has received limited attention. Although *S. enterica* (Thompson) exhibited proportional declines in viable population size upon the imposition of desiccation stress on cilantro leaves similar to two common taxa of plant colonizers, *Pantoea agglomerans* and *Pseudomonas chlororaphis* (7), in another study, a much higher proportion of cells of this species than of *P. syringae* died upon the drying of surface moisture on bean leaves (32). Thus, the fitness of *S. enterica* on leaves appears to be less than that of the more commonly recovered plant colonists that have been investigated in such detail.

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The colonization of plants by *S. enterica* can be influenced by the presence of certain other bacterial colonists of plants (5,9). The incidence of *S. enterica* colonization of produce suffering from soft rot diseases can be substantially higher than that on healthy plants. Population sizes of *S. enterica* on a variety of edible plants were as much as 10-fold higher on plants infected by *Erwinia carotovora* or *P. viridiflava* (42). However, not all plant pathogens influence the colonization of plants by *Salmonella* spp. equally; *E. chrysanthemi* facilitated the colonization of *S. enterica* much more than did *P. viridiflava* (7). Under some conditions, *S. enterica* populations were also higher on tomato plants that were co-colonized by *Xanthomonas campestris* pv. *vesicatoria*, despite the fact that no disease symptoms were present (2). Although the presence of more abundant resources in diseased tissues might have facilitated colonization by *S. enterica*, it is unclear what processes might have led to enhanced colonization in the absence of disease.

Given that *S. enterica* appears to have relatively low fitness for growth on plant surfaces, it seems likely that factors that would influence its successful immigration to plants might be of greater importance than factors that could influence its ability to multiply after immigration. Although several studies have suggested that *S. enterica* can persist on inoculated plants for extended periods of time (11,17,20), the factors that influence its survival have not been well addressed. Although *S. enterica*, like many epiphytic bacteria, can establish cellular aggregates on leaves that might be protective (8,28,30,31), such a mechanism requires that the pathogen survives and multiplies to establish such aggregates. It seems likely, therefore, that the initial survival of immigrants to leaves could be an important factor determining its presence on a plant at a location and time relevant to food safety. A recent study demonstrated that immigrant cells of nonpathogenic plant-associated bacteria survived much better when they landed as single cells onto preexisting aggregates of indigenous bacteria compared with uncolonized parts of the leaf (31). Given that *S. enterica* is apparently less fit on plants than the taxa addressed in that study, it seems likely that such a protective effect of preexisting epiphytes might be pronounced in this human pathogen. In this study, we addressed the role of other indigenous bacteria to modulate the survival of immigrant cells of *S. enterica* on leaves. Both quantitative assessments of viable population size coupled with fluorescence microscopy reveal that many bacterial species common on plants apparently change the microenvironment at local sites on the leaf in a way that makes it more conducive to the survival of immigrant cells of *S. enterica*.

MATERIALS AND METHODS

Bacterial strains and culture media. The origin and characteristics of the epiphytic bacteria *P. syringae* pv. *syringae* B728a, an *hrpJ*– mutant of *P. syringae* B728a (16); *P. fluorescens* A506; *E. herbicola* (*Pantoea agglomerans*) BRT98; and *E. herbicola* pv. *gypsophilae* 824-1 have been previously reported (23,25,35,43, 44). These bacterial strains are resistant to rifampicin (100 μg/ml) due to spontaneous chromosomal mutations. *S. enterica* subsp. *enterica* svs. Montevideo and Enteritidis have been previously described (24,40). These strains are naturally resistant to nalidixic acid (50 μg/ml). Cultures were stored frozen at –80°C in 10 mM potassium phosphate buffer (PB), pH 7.0, supplemented with 15% glycerol (vol/vol), and were routinely grown and recovered from plants on King's medium B (KB) (18) containing the fungicide natamycin (50 μg/ml).

Generation of bacterial strains expressing fluorescent protein variants. Procedures for electroporation and conjugation have been previously described (36). Plasmids were isolated by using a Qiagen Spin Miniprep kit (Qiagen Inc., Valencia, CA). The gene encoding enhanced green fluorescent protein (GFP) was isolated from plasmid pMP4655 (4). This plasmid yields constitutive GFP fluorescence in *E. herbicola* cells. Plasmid pKT-trp consists of a *gfp* marker gene whose expression is driven by the *trp* promoter from *Salmonella* sp. Typhimurium (14), and was used to yield constitutive GFP expression in *Pseudomonas syringae* and *P. fluorescens* (27). Plasmid pWM1009 harboring a consensus promoter sequence from a *Campylobacter* sp. fused to a cyan fluorescent protein *cfp* marker gene (26) was used to yield constitutive CFP expression in *S. enterica*. pKT-trp was introduced into *P. fluorescens* A506 and *P. syringae* strains by conjugation with *Escherichia coli* DH5α (pKT-trp) followed by selection on KB containing rifampicin (100 μg/ml) and kanamycin (50 μg/ml). *Erwinia herbicola* BRT98 (pMP4655) was generated by electroporation followed by selection in KB containing rifampicin (100 μg/ml) and tetracycline (10 μg/ml). *S. enterica* subsp. *enterica* (pWM1009) was generated by electroporation followed by selection in KB containing nalidixic acid (50 μg/ml) and kanamycin (50 μg/ml). No significant differences in growth rate of the strains expressing the fluorescent proteins compared with the wild-type strain were observed. Plasmid maintenance in each of the strains was determined after growth for different times without antibiotics; no apparent loss of the plasmids within 10 generations was observed (data not shown).

Plant inoculation and growth conditions. All experiments were carried out on 25-day-old lettuce plants (*Lactuca sativa* var. Romaine, 'Parris Island Cos') and 30-day-old cilantro plants (*Coriandrum sativum*). Plants were grown in a greenhouse and then inoculated with bacteria under controlled conditions in the laboratory. The population size of contaminating bacteria on these plants was $\leq\approx$100 cells/g at the time they were removed from the greenhouse, as determined by dilution plating of plating leaf washings on 10% tryptic soy agar (Difco Laboratories, Detroit) supplemented with the fungicides benomyl and natamycin (50 μg/ml each). Both the abaxial and adaxial surfaces of leaves were first inoculated with epiphytic bacteria (*P. syringae*, *P. fluorescens*, or *E. herbicola*) by spraying with a cell suspension in potassium PB (10 mM, pH. 7.0) containing 10^6 cells/ml, followed by incubation in a moist chamber at 28°C and 100% relative humidity (periodic fogging of distilled water) for 4 days to establish these species on leaves. Population size of endogenous and inoculated epiphytes were determined at 24-h intervals after recovering cells by dilution plating of leaf washing onto KB. *S. enterica* was then applied with an artist's airbrush (10^8 CFU/ml in PB) and the plants returned to the moist chamber for 24 h. *S. enterica* was also applied to control plants from the greenhouse that had not been pretreated with other bacteria, and the moistened plants were incubated for 24 h as above. All plants were then placed in a dry chamber (28°C and 30% relative humidity) for 3 days. Viable bacterial population sizes were determined by dilution plating of leaf washings similar to in other studies (32). At various times before inoculation of *S. enterica*, appropriate dilutions of leaf washates were plated onto KB to determine the sum of contaminating bacteria and the applied epiphytic species; nearly all colonies resembled the particular epiphytic species that had been inoculated. The population size of the applied epiphytic species was determined from dilution plating of leaf washings on KB containing rifampicin at 100 μg/ml and that of *S. enterica* by plating on KB containing naladixic acid at 50 μg/ml.

To determine the growth and aggregation status of *S. enterica* cells on leaves, the abaxial and adaxial surfaces of lettuce leaves were inoculated with a suspension of *S. enterica* cells (4×10^4 cells/ml in PB) using an artist's airbrush. Plants were then incubated in a moist chamber as above for 7 days and population sizes determined by dilution plating as above.

To determine the pathogenicity of *P. syringae* B728a strains to lettuce, the abaxial surface of leaves were inoculated with 100 μl of a suspension of bacteria at different concentrations (10^6, 10^7,

and 10^8 cells/ml) in 10 mM PB using a syringe. Symptoms were visualized after both 1 and 3 days.

Sample preparation for microscopy. To study the formation of cell aggregates of *S. enterica*, four randomly chosen leaves were harvested at various times after inoculation, and four sections of 1.5 by 1.5 mm, equivalent to 0.55 mg of leaf material exposing both the abaxial and adaxial surfaces, were cut from each leaf. Four images were then taken for each section and visualized as below. The viability of *S. enterica* cells recovered 24 h after inoculation onto moist plants as well as 24 and 72 h after plants were subsequently allowed to dry was assessed using similar methods; five images were taken for each of four sections removed from each of four leaves. Circular segments of ≈1 cm in diameter were randomly cut from leaves and placed on top of 100 µl of melted water agar (1%) on a microscope slide to stabilize the leaf segments and to ensure a flat surface for microscopic observations (28). After the agar had solidified (≈20 s), 10 µl of a solution of propidium iodide (10 µg/ml) in Polymount was placed on the center of a coverslip, which was then gently applied to the leaf segment. The mountings were kept in the dark at room temperature for 10 min and then observed by epifluorescence microscopy.

Epifluorescence microscopy. Samples were observed by epifluorescence microscopy using an Axiophot Zeiss microscope equipped with either ×10/0.30, ×20/1.30, ×40/0.75, or ×100/1.30 numerical-aperture Plan Neofluar objectives (Zeiss Inc., Oberkochen, Germany). A CFP/yellow fluorescent protein 51017 filter set (Chroma Technology Corp., Brattleboro, VT) was used to visualize green and cyan fluorescence in the same field of view and, thus, to enumerate the number of GFP-marked and CFP-marked cells present in a given aggregate. A 61000v2 DAPI/FITC/TRITC filter (Chroma Technology Corp.) was used to visualize red propidium iodide fluorescence indicative of cell death. This filter permitted simultaneous visualization of dead and living (GFP or CFP fluorescent) cells. Images were captured with an Optronics DEI-750 video camera, transferred to a personal computer platform, and processed with Adobe PhotoShop software (Adobe Systems Incorporated). Image processing was carried out by combining overlaying the images that had been separately obtained with each filter. In the images obtained with the CFP filter, cell death was observed as a decrease in fluorescence intensity (blue or green). Cell death of particular inoculated bacterial strains was determined by combining the images obtained with the 4′,6-diamidino-2-phenylindole (DAPI) and CFP filters, which enabled distinguishing dead endogenous bacteria from dead inoculated bacteria.

Quantification of monospecific and mixed bacterial aggregates. The entire surface of each leaf segment was extensively scanned at different magnifications in order to locate individual *S. enterica* cells as well as *S. enterica* cells within monospecific or mixed species aggregates (two or more touching cells was considered an aggregate). Two images were captured for each field of view (one image with the CFP filter and another with the DAPI filter). All images were captured at a magnification of ×500 and processed at a resolution of 300 pixels/in.^{2} using Adobe PhotoShop software. Individual *S. enterica* cells (live or dead) were enumerated visually. One *S. enterica* cell is composed of 67 pixels. The number of cells in a given aggregate was calculated based on the area of that object, assuming that they formed a monolayer as in other studies (29,30).

Statistics. All experiments were repeated at least twice, with three replications for each experiment. Data management and computation were performed by using Microsoft EXCEL. Descriptive statistics and analysis of variance of measurements of the proportion of living cells were all performed with SYSTAT (Systat Software, Inc., Chicago) using a significance level of $P = 0.05$. The standard errors of all estimates of the mean of dependent variables are shown.

RESULTS

S. enterica sv. Montevideo exhibited lower epiphytic fitness on the surface of romaine lettuce leaves than did the mixture of endemic bacteria that occurred on these greenhouse-grown plants (Fig. 1). Whereas the population size of the inoculated *S. enterica* strain as well as culturable endemic bacteria were each initially ≈10^4 cells/fresh weight of tissue, the mixed population of endemic bacteria increased to over 10^7 cells/g while that of *S. enterica* increased to only ≈10^6 cells/g after ≥3 days of incubation (Fig. 1). Because the *S. enterica* strain harbored a plasmid conferring constitutive GFP fluorescence, its total population size was also measured by fluorescence microscopy of cells recovered from the leaf. The total *S. enterica* population sizes determined by fluorescence microscopy were very similar to those of culturable population sizes, suggesting that nearly all cells were alive on such moist plants and that the *gfp* marker gene was maintained on plants.

The influence of preexisting bacterial residents on romaine lettuce plants to the survival of immigrant cells of *S. enterica* sv. Montevideo was assessed by comparing the number of viable cells of this species when applied to plants that had been previously inoculated with one of several different common epiphytic bacteria with that recovered from plants lacking large preexisting epiphytic populations after both were subjected to desiccation stress after inoculation. Each of the bacterial strains (*P. syringae* B728a, *E. herbicola* pv. *gypsophila* 824-1, *E. herbicola* BRT98, and *P. fluorescens* A506) (hereafter referred to as indigenous epiphytic strains) increased in population size from ≈10^5 cells/g shortly after inoculation to ≈10^7 cells/g within the 4-day incubation period on plants kept moist after inoculation (Fig. 2). *S. enterica* was then challenge inoculated onto these preinoculated plants or on control plants that had not been precolonized by large bacterial populations and the plants kept moist for 24 h. After this 24-h moist incubation period, plants were allowed to dry for an additional 3 days to impose desiccation stress on the bacteria. The numbers of culturable *S. enterica* cells (≈10^6 cells/g) exhibited little change during this 24-h moist incubation period (Fig. 2). However, the numbers of culturable cells decreased rapidly on plants as leaf surface moisture dried. Viable population sizes of *S. enterica* decreased 10- to 100-fold within 3 days of the imposition of desiccation stress (Fig. 2). Importantly, the loss of viability of *S. enterica* was much less, in all cases, on those plants

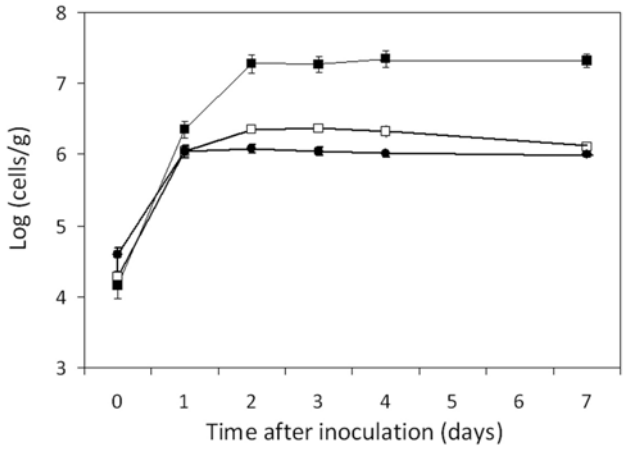

Fig. 1. Culturable population size of a green fluorescent protein (*gfp*)-marked *Salmonella enterica* sv. Montevideo on the surface of romaine lettuce leaves kept moist after inoculation. Viable population size was determined by dilution plating (open squares) and the total numbers of this strain determined by fluorescence microscopy of leaf washings (filled circles). The population size of endemic bacteria on uninoculated plants was determined by dilution plating (filled squares). Vertical bars represent the standard error of the mean of log-transformed population size.

having a large preexisting population of a given epiphytic bacterial species compared with those on control plants lacking such large population sizes at the time of inoculation of *S. enterica* (Fig. 2). For example, culturable population sizes of *S. enterica* on plants preinoculated with *P. syringae* or either of the *E. herbicola* strains was ≥10-fold higher than that on control plants that were not precolonized by these indigenous bacteria when assessed 24 or 72 h after the imposition of desiccation stress (Fig. 2). Precolonization with *E. herbicola* BRT98 enhanced the survival of *S. enterica* the most while the protection conferred by *P. fluorescens* A506 was two- to threefold less (Fig. 2). Although no evidence of in vitro antagonism was observed between either *P. syringae* or either strain of *E. herbicola* and any strain of *S. enterica*, modest antibiosis of *P. fluorescens* A506 to some strains of *S. enterica* was observed (Supplemental Figure 1).

Although the population sizes of the indigenous epiphytic bacterial strains that were preinoculated onto plants were usually much higher than those of *S. enterica* after desiccation stress, the ability of these strains to suppress the death of *S. enterica* appeared unrelated to their desiccation stress tolerance. For example, whereas the numbers of viable cells of *E. herbicola* BRT98 72 h after the imposition of desiccation stress were much higher than those of *P. syringae,* both strains conferred similar protection to lethal desiccation stress of *S. enterica* (Fig. 2). Because the indigenous epiphytic bacterial strains preinoculated onto plants had achieved much higher population sizes before the imposition of desiccation stress, their higher population sizes after plants had dried was largely because of their larger initial populations, and they exhibited a similar proportional loss in viability as did *S. enterica* on plants that had not been preinoculated.

To verify that the protective effect of indigenous bacteria on desiccation stress tolerance of *S. enterica* was not a strain-specific interaction with *S. enterica* sv. Montevideo, the effects of these four indigenous bacterial species on the survival of *S. enterica* sv. Enteriditis was assessed in similar studies. Remarkably similar patterns of protection of *S. enterica* sv. Enteriditis were conferred by these four strains as were seen for sv. Montevideo (Fig. 3). The death of *S. enterica* sv. Enteriditis was reduced ≥10-fold on plants preinoculated with either *P. syringae* or either of the two *E. herbicola* strains, while lesser protection against desiccation stress was conferred by *P. fluorescens* A506 (Fig. 3). Likewise, the presence of *P. syringae* conferred protection to *S. enterica* sv. Enteriditis despite the fact that its own population size decreased much more than either of the other three indigenous strains (Fig. 3).

To verify that the protective effect of indigenous epiphytic bacterial species on the desiccation stress tolerance of *S. enterica* was not a plant-species-specific phenomenon restricted to romaine lettuce, the survival of *S. enterica* sv. Montevideo was compared on leaves of cilantro that either had or lacked large populations of the epiphytes. As observed on romaine lettuce, the population size of *S. enterica* preincubated with either *P. syringae* or one of the two *E. herbicola* strains was nearly 10-fold higher than that on plants that did not harbor large numbers of indigenous bacteria (Fig. 4). Also as seen on romaine lettuce, the apparent protective effect of *P. fluorescens* A506 was noticeably less than that of either of the other three epiphytic strains (Fig. 4). Likewise, *P. syringae* exhibited less tolerance of desiccation stress than did any of the three other epiphytic bacteria; the population size of *P. syringae* was as much as 10-fold lower than that of the other bacteria when assessed 72 h after the imposition of desiccation stress (Fig. 4). Regardless of whether it was inoculated onto precolonized or control plants, *S. enterica* exhibited less ability to tolerate desiccation stress on cilantro than did either of the *E. herbicola* strains or *P. fluorescens* A506 (Fig. 4). Additionally, no net increase in population size of *S. enterica* occurred on cilantro during a 24-h moist incubation period after inoculation onto either

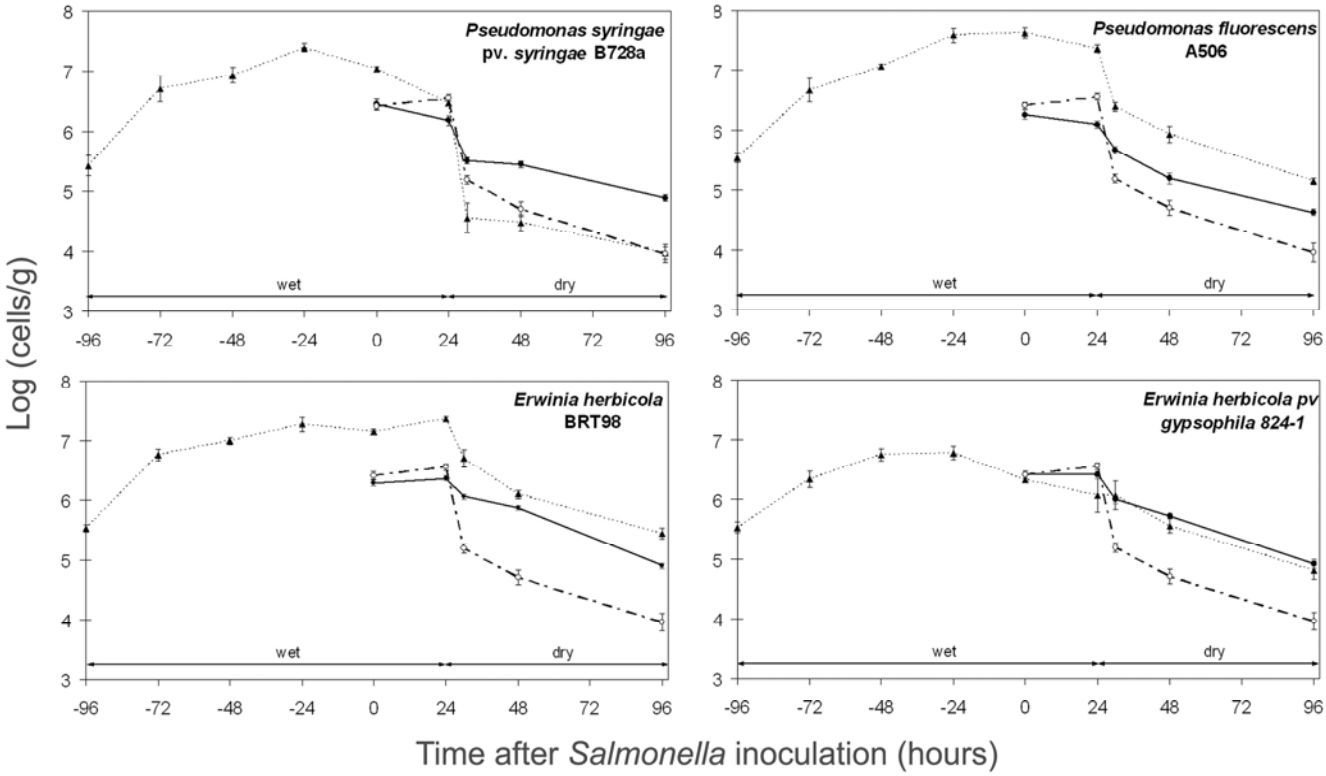

Fig. 2. Viable population size of different bacterial strains at various times after inoculation onto romaine lettuce leaves kept moist for 4 days (dotted line, filled triangles). After this 4-day preincubation period (denoted as 0 h after inoculation), these plants that had been precolonized with a given bacterial strain (solid line, filled circles) or other plants that had not been previously inoculated (dashed line, open circles) were inoculated with *Salmonella enterica* sv. Montevideo and kept moist for an additional 24 h before plants were then allowed to dry. Culturable *S. enterica* populations were determined at various times after inoculation onto the plants that had been preinoculated with a given bacterial strain (filled circles) or which had not been preinoculated (open circles). Vertical bars represent the standard error of the mean of log-transformed population size.

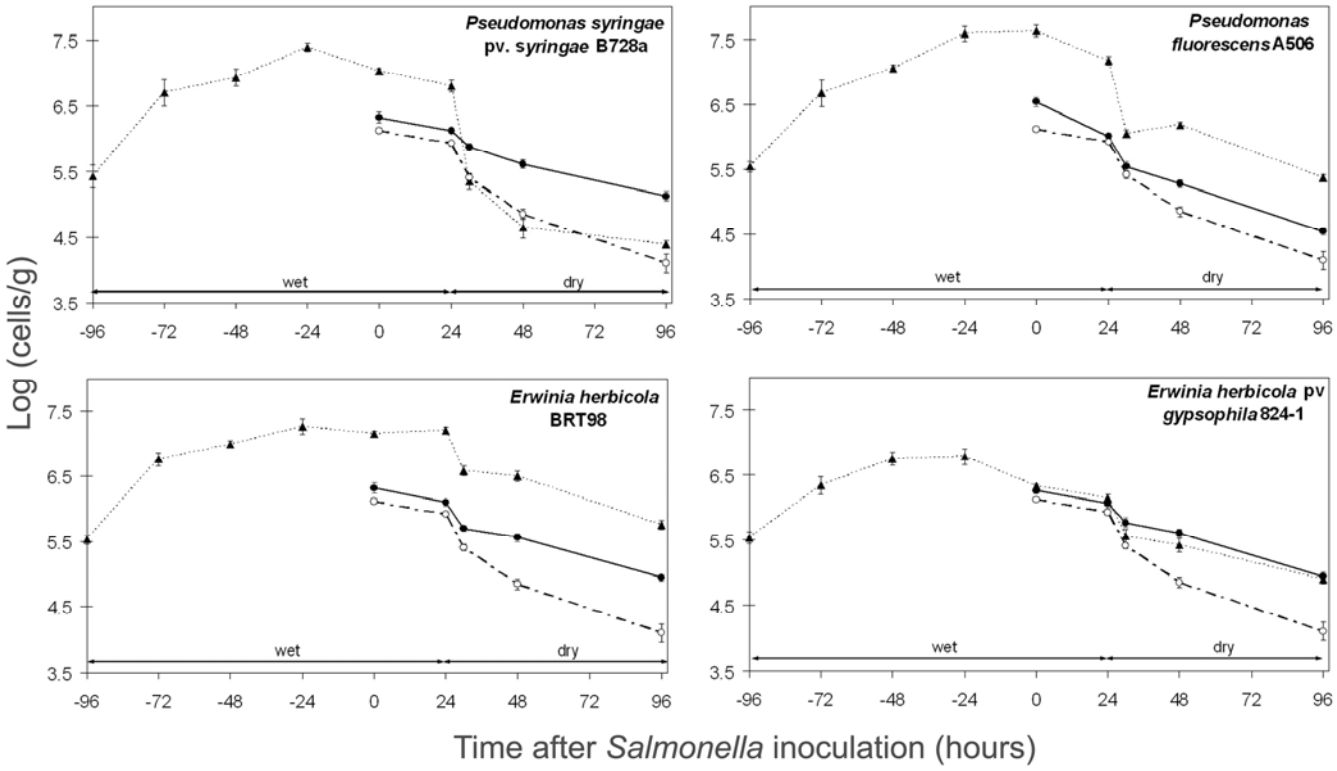

Fig. 3. Viable population size of different bacterial strains at various times after inoculation onto romaine lettuce leaves kept moist for 4 days (dotted line, filled triangles). After this 4-day preincubation period (denoted as 0 h after inoculation), these plants that had been precolonized with a given bacterial strain (solid line, filled circles) or other plants that had not been previously inoculated (dashed line, open circles) were inoculated with *Salmonella enterica* sv. Enteriditis and kept moist for an additional 24 h before plants were then allowed to dry. Culturable *S. enterica* populations were determined at various times after inoculation onto the plants that had been preinoculated with a given bacterial strain (filled circles) or which had not been preinoculated (open circles). Vertical bars represent the standard error of the mean of log-transformed population size.

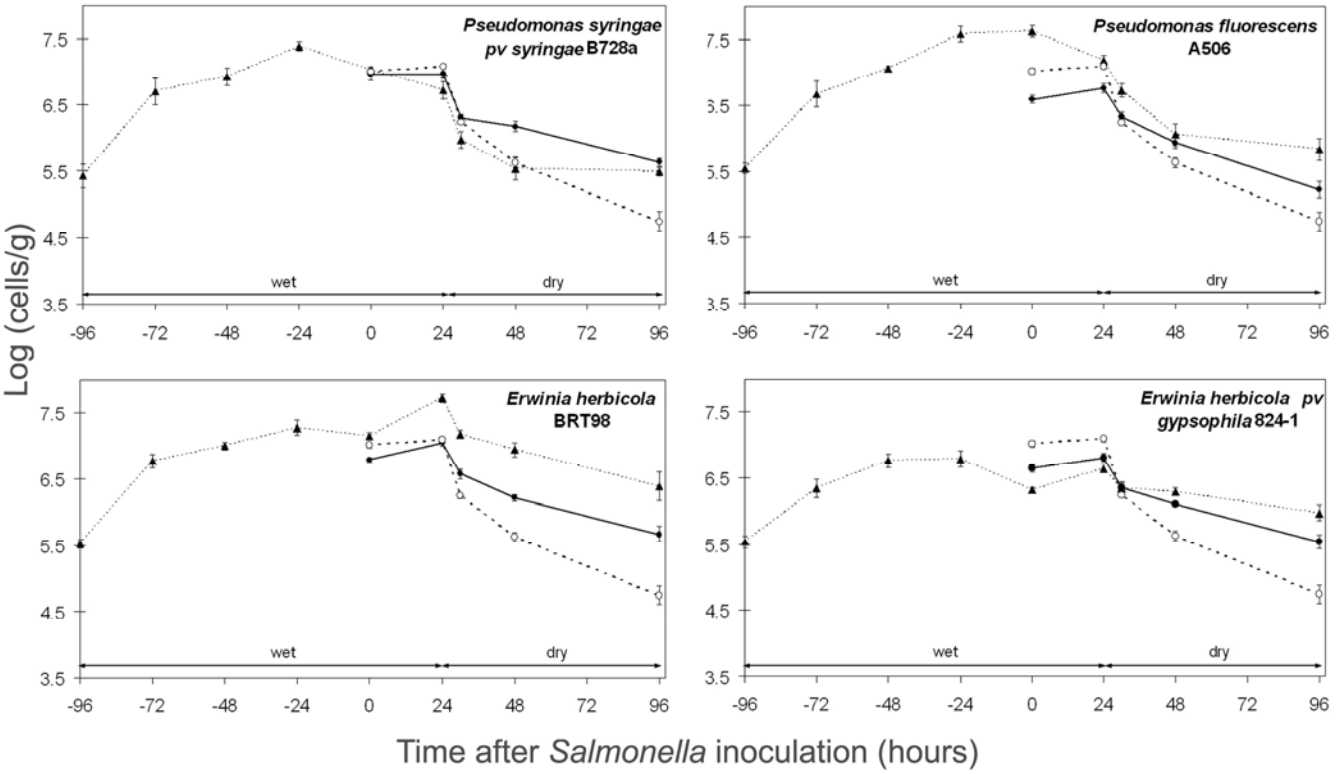

Fig. 4. Viable population size of different bacterial strains at various times after inoculation onto cilantro leaves kept moist for 4 days (dotted line, filled triangles). After this 4-day preincubation period (denoted as 0 h after inoculation), these plants that had been precolonized with a given bacterial strain (solid line, filled circles) or other plants that had not been previously inoculated (dashed line, open circles) were inoculated with *Salmonella enterica* sv. Montevideo and kept moist for an additional 24 h before plants were then allowed to dry. Culturable *S. enterica* populations were determined at various times after inoculation onto the plants that had been preinoculated with a given bacterial strain (filled circles) or which had not been preinoculated (open circles). Vertical bars represent the standard error of the mean of log-transformed population size.

precolonized or control plants. When assessed 72 h after the initiation of desiccation stress, the relative viable population size of the two different *S. enterica* strains on either romaine lettuce or cilantro that had been precolonized with a given resident epiphytic strain was similar; on a given plant species, its viable population size was similar on plants pretreated with either *P. syringae* or either of the *E. herbicola* strains and greater than that on plants pretreated with *P. fluorescens* which, in turn, was substantially higher than that on plants lacking a large indigenous epiphytic bacterial community.

We considered that the protective effect of *P. syringae* on desiccation stress tolerance of *S. enterica*, despite the fact that it itself did not survive well on either romaine lettuce or cilantro, might be associated with virulence traits that it might exhibit. Although *P. syringae* B728a is not a pathogen of romaine lettuce (only a hypersensitive reaction was seen within 24 h of inoculation of plants with high but not low concentrations of the wild-type strain but not the *hrpJ* mutant), it seemed possible that it could have influenced the plant in a way that could have reduced the stress that *S. enterica* would have experienced. As a test of this hypothesis, we compared the desiccation stress tolerance of *S. enterica* sv. Montevideo on romaine lettuce precolonized by either a wild-type or a nonpathogenic *hrpJ*– mutant of *P. syringae*. The viable population size of *S. enterica* on plants 72 h after plants were allowed to dry was more than 10-fold higher on plants precolonized by either the wild-type or *hrpJ*– *P. syringae* strain than on control plants lacking such epiphytic colonists (Fig. 5). The population sizes of *S. enterica* on plants pretreated with the two *P. syringae* strains did not differ (Fig. 5). Curiously, whereas the proportional decrease in viable population size of the wild-type *P. syringae* strain upon drying of the leaves was similar to that of *S. enterica* on control leaves, the *hrpJ*– mutant exhibited a greater proportional decrease than did the wild-type *P. syringae* strain (Fig. 5). Thus, although the type III secretion system (and perhaps certain effectors produced by *P. syringae*) contributes to its epiphytic fitness on the non-host plant romaine lettuce, as has also been seen on the host plant bean (16), it apparently does not mediate the protective effect of *P. syringae* to cells of *S. enterica*.

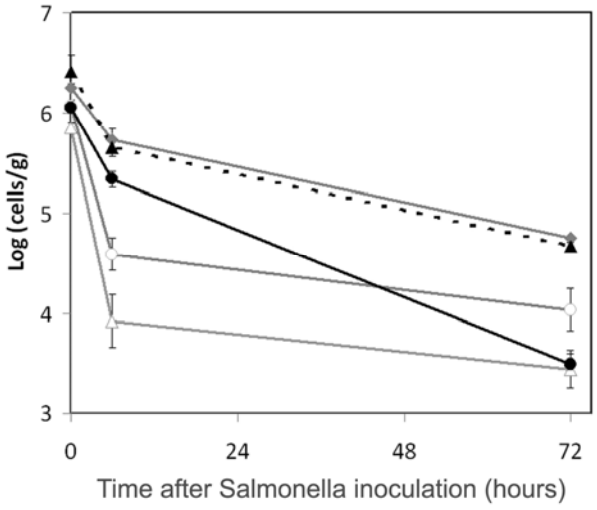

Fig. 5. Culturable population size of *Salmonella enterica* sv. Montevideo on romaine lettuce leaves at various times after it was applied to plants that had been inoculated 4 days previously with either wild-type *Pseudomonas syringae* strain B728a (diamonds) or an *hrpJ*– mutant of strain B728a (filled triangles) and on plants not previously inoculated (filled circles) and then allowed to dry shortly after inoculation with *S. enterica*. The time after inoculation with *S. enterica* is shown on the abscissa. Shown also is the population size of strain B728a (open circles) and of the *hrpJ*– mutant (open triangles) at various times after plants were allowed to dry. Vertical bars represent the mean of log-transformed population sizes.

Because *S. enterica* has been shown to produce cellular aggregates under some conditions on plants (8), and because the survival of cellular aggregates of some bacterial species such as *P. syringae* was greater than that of more solitary cells (28), we determined to what extent the survival of *S. enterica* under desiccation conditions in this study could be attributed to its formation of cellular aggregates. Dispersed suspensions of a GFP- or CFP-expressing *S. enterica* strain were applied to romaine lettuce leaves using an artist's paint brush that resulted in the deposition primarily of individual cells (data not shown), and the plants were then incubated under moist conditions that allowed bacterial growth for ≥1 days. Cellular aggregates visualized using epifluorescence microscopy were not large, consistent with the relatively restricted growth exhibited by *S. enterica* on romaine lettuce, and were most commonly observed in the grooves between plant cells (Fig. 6). The numbers of CFP-labeled S. *enterica* cells that occurred individually or in groups of two or more cells was determined from analysis of digital images obtained during epifluorescence microscopy. When examined after 48 h of incubation on moist leaves, a majority of *S. enterica* cells were in small cellular aggregates (Fig. 7); ≈75% of the CFP-labeled *S. enterica* cells were in aggregates of two or more cells. Both the proportion of cells found in cellular aggregates as well as the total number of cells (either in aggregates or solitary cells) was very similar when assessed either 1 or 3 days after the imposition of desiccation stress (Fig. 7), suggesting that little or no cellular growth occurred after plants were allowed to dry. The death of the CFP-labeled *S. enterica* cells with increasing duration of drying

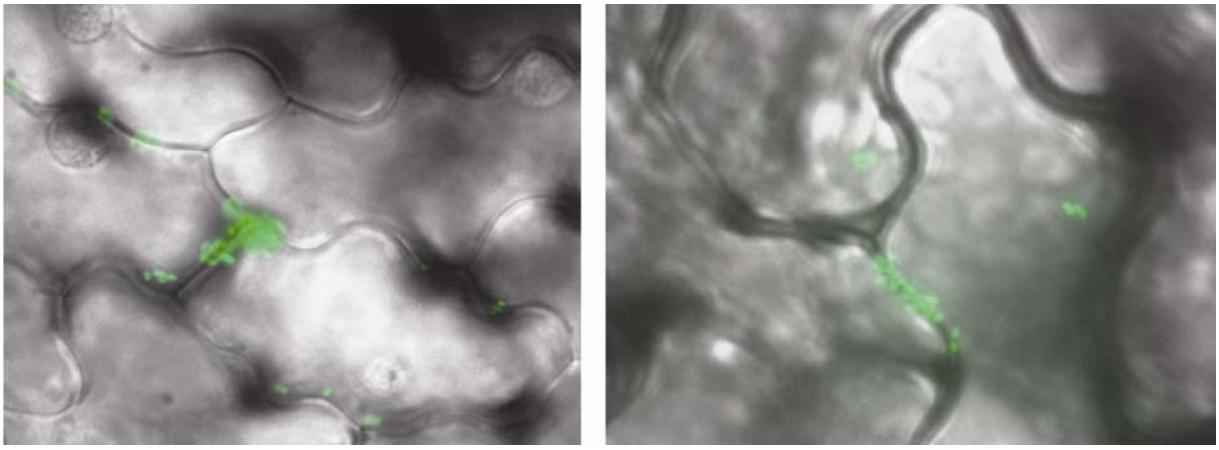

Fig. 6. Aggregates of a green fluorescent protein (*gfp*)-marked strain of *Salmonella enterica* sv. Montevideo on the surface of romaine lettuce leaves visualized by fluorescence microscopy.

was determined by simultaneously assessing CFP fluorescence and the red fluorescence associated with propidium iodide staining of cells directly on leaves by epifluorescence microscopy. Only a very small proportion of either solitary or aggregated CFP-labeled cells on leaves that had been wet for 48 h were stained by propidium iodide and, thus, presumed dead (Fig. 7). The proportion of cells that stained with propidium iodide increased with increasing duration of desiccation stress on dry leaves but was always much lower for aggregated than solitary cells (Fig. 7). Thus, under conditions in which cellular aggregates of *S. enterica* formed on leaves, such aggregations are less susceptible to death by dehydration. However, because a very high percentage of solitary cells had succumbed to desiccation stress by 3 days of exposure on dry leaves, solitary immigrant cells of *S. enterica* are apparently highly susceptible to death by dehydration.

Because little apparent multiplication of *S. enterica* occurred in the 24-h interval after they were inoculated onto moist leaves that were already supporting large indigenous epiphytic bacterial

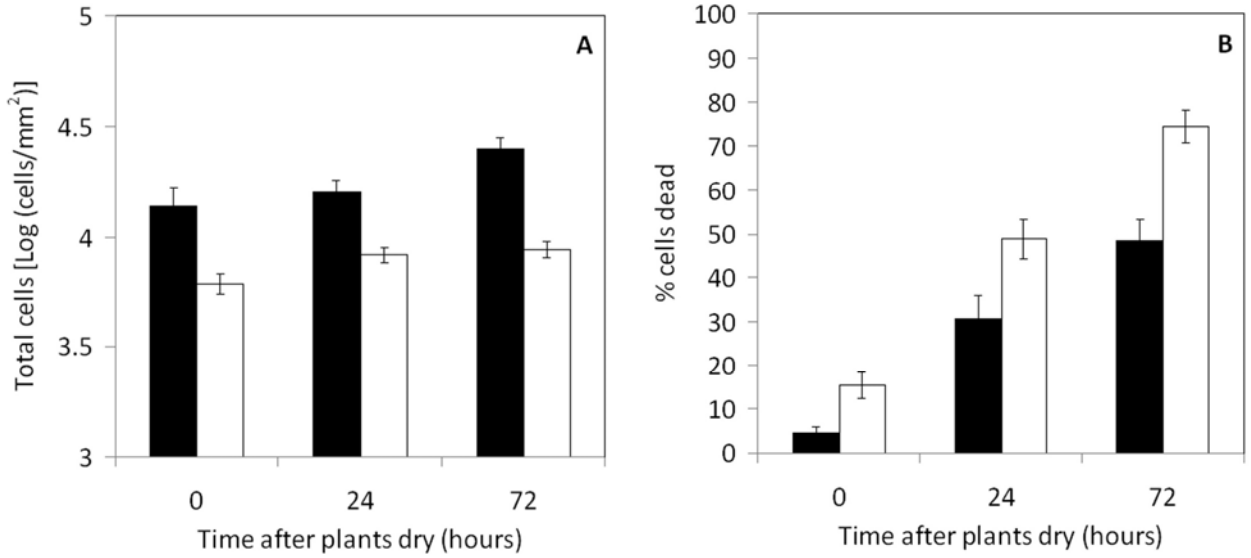

Fig. 7. A, Total number of cells of a green fluorescent protein (*gfp*)-marked strain of *Salmonella enterica* sv. Montevideo determined by fluorescence microscopy that occurred in cellular aggregates of two or more cells (dark bars) or as solitary cells (white bars) on the surface of romaine lettuce at various times after plants were allowed to dry subsequent to a 24-h initial moist incubation period after inoculation. **B,** Percentage of cells of *S. enterica* in cellular aggregates (dark bars) or solitary cells (white bars) that were dead as determined by propidium iodide staining and fluorescence microscopy when incubated under dry conditions for various times as described above. Vertical bars represent the standard error of the determination of mean log-transformed population size or percentage of dead cells.

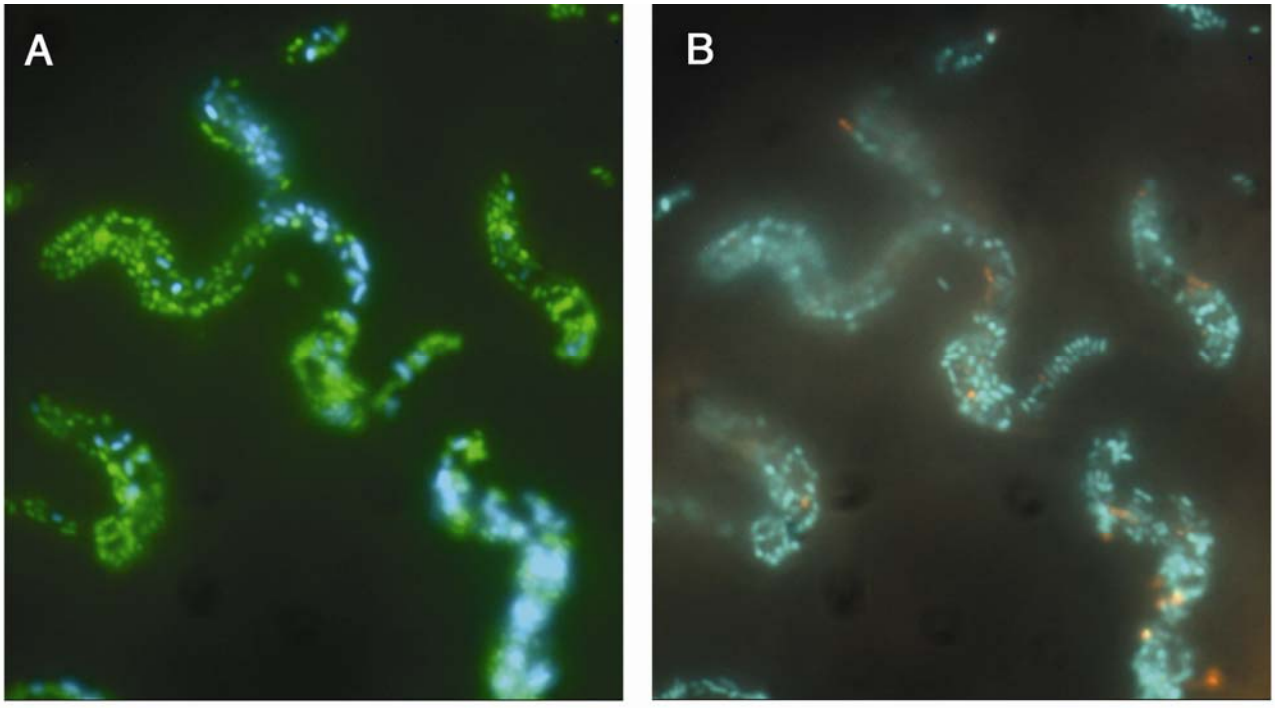

Fig. 8. Co-aggregation of cells of green fluorescent protein (*gfp*)-marked *Erwinia herbicola* strain BRT98 and a cyan fluorescent protein (*cfp*)-marked strain of *Salmonella enterica* sv. Montevideo on the surface of romaine lettuce leaves that had been incubated for 24 h under moist conditions and then an additional 72 h under dry conditions after inoculation with *S. enterica* before being visualized using fluorescence microscopy using an enhanced CFP filter set to distinguish *gfp* and *cfp* fluorescence (**A**). Cells that had died during the dry incubation were identified by their red fluorescence associated with propidium iodide staining when visualized with a 4′,6-diamidino-2-phenylindole filter set (**B**).

populations, we hypothesized that the surviving immigrant cells were located in preexisting cellular aggregates of the resident bacteria. We further hypothesized that the protective effect of preexisting cellular aggregates on romaine lettuce leaves would be restricted to those immigrant cells that were coincident with the cellular aggregates. Thus, we assessed the spatial relationships and viability of both GFP-marked strains of resident epiphytic bacteria that were allowed to precolonize leaves as well as a CFP-marked strain of *S. enterica* that immigrated largely as solitary cells during inoculation with an artist's airbrush. When assessed 24 h after inoculation and subsequent incubation on moist leaves, similar numbers of *S. enterica* cells were found as homogeneous aggregates, as solitary cells, and as individual cells in mixed aggregates with a given resident epiphytic bacterium such as *E. herbicola* BRT98 (Figs. 8 and 9). Most *S. enterica* cells coincident with aggregates of a given resident epiphytic bacterium occurred as solitary cells (Fig. 8), suggesting that relatively little multiplication of *S. enterica* had occurred on leaves that were precolonized by a given resident bacterial strain. The death of immigrant *S. enterica* cells upon drying of the leaves was assessed by propidium iodide staining. CFP-labeled *S. enterica* cells that were stained by propidium iodide could be distinguished from those of the GFP-labeled resident epiphytes (Fig. 8). Although nearly all cells of *S. enterica* present on moist plants before they dried did not stain with propidium iodide and, thus, were apparently alive, a high proportion of solitary *S. enterica* cells had died by 1 or 3 days on dry leaves (Fig. 9). Importantly, the proportion of *S. enterica* cells that had formed homogeneous aggregates or which had been found in mixed aggregates with *E. herbicola* BRT98 that had succumbed to desiccation stress by 1 or 3 days was much lower than the solitary cells (Fig. 9). The frequency of dead individual *Salmonella* cells that were co-incident with aggregates of *E. herbicola* BRT98 was less than that of homogeneous *S. enterica* aggregates (Fig. 9). Significantly, the proportion of individual cells of *S. enterica* coincident with aggregates of *P. fluorescens* A506 that had stained with propidium iodide after either 1 or 3 days of exposure to desiccation stress on dry leaves was similar to that of solitary cells (Fig. 10). In contrast, the proportion of cells in homogeneous aggregates of *S. enterica* that were stained by propidium iodide after either 1 or 3 days of exposure to desiccation stress on dry leaves was lower than those of individual cells or cells in a mixed aggregate with *P. fluorescens* (Fig. 10). Curiously, a relatively high percentage of *S. enterica* cells coincident with *P. fluorescens* stained with propidium iodide even on plants that did not experience drying, suggesting that this strain had damaged *S. enterica* directly.

DISCUSSION

The survival of *S. enterica* on the leaves of edible plants is clearly context dependent because it was strongly influenced by the presence of resident bacteria in their vicinity. Interestingly, such interactions could either enhance or inhibit the survival of these immigrant cells in a strain-dependent manner. The survival of *S. enterica* was strongly increased when immigrant cells were coincident with several strains of common epiphytic species such as *P. syringae* and *E. herbicola*, suggesting that they directly enhance survival of this human pathogen. The much reduced beneficial effect of aggregates of *P. fluorescens* A506 was apparently due to negative interactions that occurred between the two strains that overwhelmed a physical environment that might otherwise have been conducive for the survival of *S. enterica*. Given the diverse species that appear to be protective of *S. enterica*, we presume that most bacteria that are not directly inhibitory toward *S. enterica* will confer some protection to this human pathogen. It seems likely that the indirect benefits to *S. enterica* that are conferred by prior colonization of leaves by a variety of bacteria is due to their modification of the microenvironment that cells experience on the leaf surface environment. Copious extracellular polysaccharide (EPS) is often observed around epiphytic bacteria on leaves (21,22). Such EPS is protective of the bacterial species such as *P. syringae* that produce it (46), and it is likely that it would be protective of other bacterial cells that encounter it. It is possible that other bacterial products such as biosurfactants or even phytotoxins could alter the local environment of the plant in a way that would stimulate not only the producer's survival but also that of other bacteria as well. Thus, epiphytic bacterial species such as *E. herbicola* that are

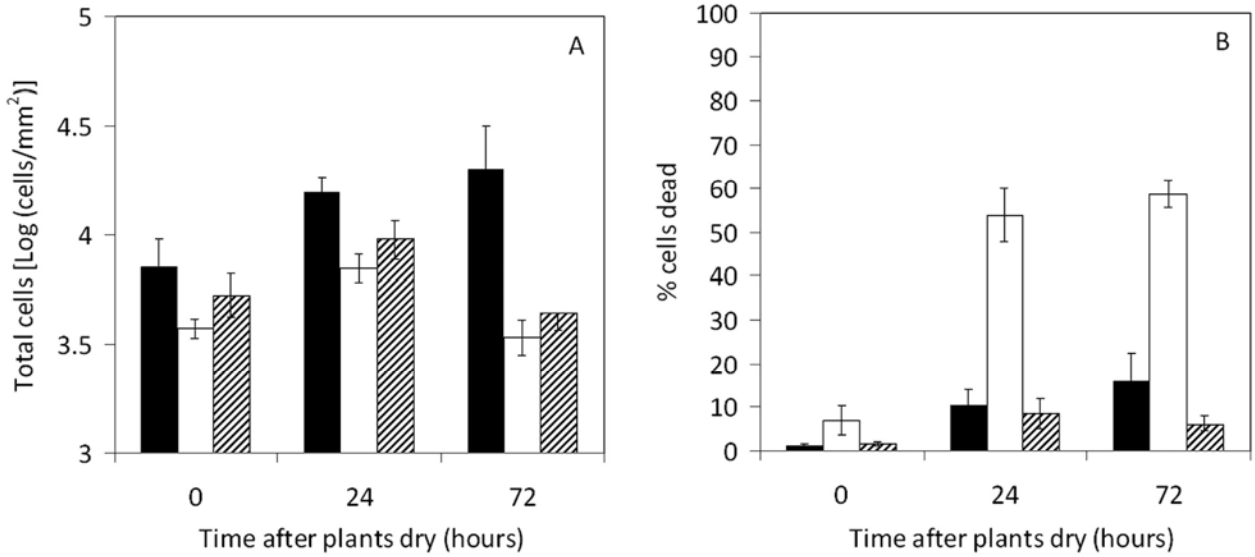

Fig. 9. A, Total number of cells of a cyan fluorescent protein (*cfp*)-marked strain of *Salmonella enterica* sv. Montevideo that occurred in homogeneous cellular aggregates of two or more cells (dark bars), as solitary cells (white bars), or as individual cells in a mixed aggregate with a green fluorescent protein (*gfp*)-marked strain of *Erwinia herbicola* BRT98 (striped bars) determined by fluorescence microscopy on the surface of romaine lettuce at various times after plants were allowed to dry subsequent to a 24-h initial moist incubation period after inoculation with *S. enterica*. **B,** Percentage of cells of *S. enterica* present in homogeneous cellular aggregates (dark bars), as solitary cells (white bars), or as individual cells in a mixed aggregate with a *gfp*-marked strain of *Erwinia herbicola* BRT98 (striped bars) that were dead as determined by propidium iodide staining and red fluorescence when incubated under dry conditions for various times as described above. Vertical bars represent the standard error of the determination of mean log-transformed population size or percentage of dead cells.

common colonists of plants presumably modify the leaf surface environment in a manner that benefits not only them but other species that are coincident. Indeed, the colonization of plants by *P. agglomerans* was shown to protect immigrant cells of not only of this species but also of *P. fluorescens* (31).

Factors that influence the survival of *S. enterica* on plants might be particularly important in its epidemiology on plants. Animal hosts are the probable source of these strains (33). Many possible mechanisms exist by which *S. enterica* could be transferred to plants, including contaminated water, application of pathogen-infested animal waste to soil, direct deposition from animal feces, and perhaps even by vectoring by insects or direct aerosol deposition (5,17,33,34). *S. enterica* appears to be less fit than many other bacteria normally found on leaves, and capable of abundant growth only under the conditions of high temperature and the presence of moisture, conditions unlikely to be commonly found on plants (5). For this reason, processes that influence the survival of immigrant inoculum probably play a prominent role in determining the presence of viable cells of this pathogen on plant surfaces. The finding here that the presence of a variety of common epiphytic bacteria on edible plants facilitates the survival of *Salmonella* spp. during dry environmental conditions to which it might normally succumb suggests that the normal microflora of plants might be considered a risk factor for human exposure to *S. enterica*. That is, plants harboring relatively low population sizes of epiphytic bacteria might be expected to be less capable of supporting the survival of immigrant cells of *Salmonella* should contamination occur. Although the study did not address the population size-dependent protection of *S. enterica* by epiphytic bacteria, the finding that protection appeared to be restricted only to those immigrant cells that had merged with aggregates of existing epiphytes (Figs. 9 and 10) suggests that high cell numbers, which would increase the numbers or sizes of cellular aggregates, would favor such a protective effect. Thus, we would expect that those plants with the highest population sizes of epiphytic bacteria would also be the most protective of immigrant *S. enterica*. Furthermore, cultural practices such as overhead irrigation or other procedures that would favor the development of large epiphytic bacterial populations might also enhance the likelihood that such plants would support viable populations of *S. enterica* should contamination events occur.

The wet conditions to which the plants were exposed after *S. enterica* was applied apparently maximized the likelihood that these immigrant cells would eventually be co-localized with preexisting bacterial epiphytes. In a previous study in which cells of various bacterial species were atomized onto leaves in a similar manner to that used here, <1% of the cells were coincident with preexisting epiphytes (31). In that study, leaves remained dry after application of the immigrant bacteria, unlike in this study, where leaves were wetted for 24 h after inoculation of *S. enterica*. In the current study, nearly half of the cells of *S. enterica* were co-localized with a given preexisting epiphyte (Fig. 9). Therefore, the method by which immigrant cells arrive at a leaf surface may influence both the likelihood that they could become coincident with existing epiphytic bacteria and, thus, their likelihood of surviving subsequent stressful conditions. Thus, it seems likely that cells of *S. enterica* that would immigrate to leaves in contaminated water or by rain splash would have a maximal likelihood of encountering preexisting bacterial colonists. It is perhaps not coincidental that leaf locations such as the grooves between epidermal cells are the sites where bacterial aggregates predominantly occur. Such sites are likely to be where water collects and where it is last to evaporate during the drying process (21,22). Thus, both the original colonists of a leaf and subsequent immigrants such as *S. enterica* are both likely to be found at such sites, largely as a result of the physical process of water movement on the leaf. It seems likely that, if *S. enterica* immigrated to a leaf in a process that did not involve water, the leaf would have to subsequently be wetted by rain or dew after the immigration event but before the death of the immigrant cells for such cells to benefit from habitat modification that might have been conferred by preexisting epiphytes; lacking such redistribution, it is unlikely that immigrant cells would encounter preexisting epiphytes by chance and, thus, they would not benefit from any habitat modification that they might have conferred.

It seems likely that many epiphytic bacterial species would be protective of *S. enterica* on leaves. Although three of the four strains tested for such an effect in the study were strongly

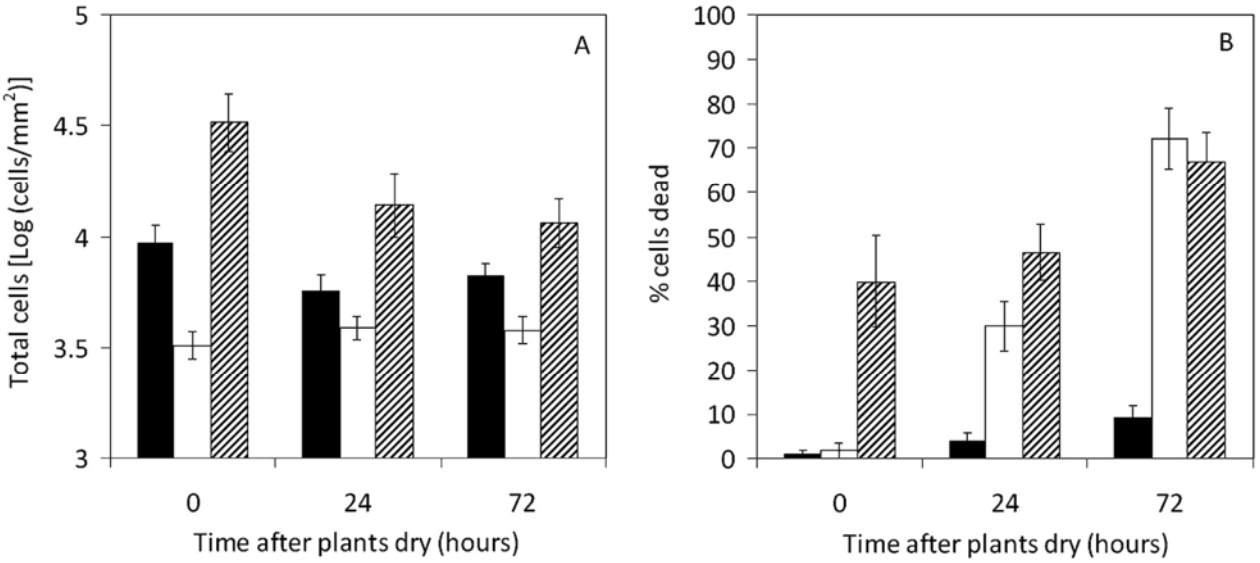

Fig. 10. A, Total number of cells of a cyan fluorescent protein (*cfp*)-marked strain of *Salmonella enterica* sv. Montevideo that occurred in homogeneous cellular aggregates of two or more cells (dark bars), as solitary cells (white bars), or as individual cells in a mixed aggregate with a green fluorescent protein (*gfp*)-marked strain of *Pseudomonas fluorescens* A506 (striped bars) determined by fluorescence microscopy on the surface of romaine lettuce at various times after plants were allowed to dry subsequent to a 24-h initial moist incubation period after inoculation with *S. enterica*. **B,** Percentage of cells of *S. enterica* present in homogeneous cellular aggregates (dark bars), as solitary cells (white bars), or as individual cells in a mixed aggregate with a *gfp*-marked strain of *Erwinia herbicola* BRT98 (striped bars) that were dead as determined by propidium iodide staining and red fluorescence when incubated under dry conditions for various times as described above. Vertical bars represent the standard error of the determination of mean log-transformed population size or percentage of dead cells.

protective, even *P. fluorescens* A506, which exhibited in vitro antibiosis, damaging at least some cells of *S. enterica* (Fig. 10), conferred at least modest survival of the population as a whole (Figs. 2 to 4). It seems possible that the spatial extent of any protective habitat modification that might have been conferred by this strain would have been greater than the region in which antibiosis is operative. It is significant that the surviving population size of an inoculated strain of *P. syringae* was highly positively correlated with the population size of indigenous bacteria on the same plants under field conditions (19). Such plants presumably harbored a wide variety of different bacterial taxa, some of which undoubtedly were inhibitory toward *P. syringae*. Thus, that study provides evidence that indigenous bacteria might generally favor the survival of immigrant bacteria and that such a phenomenon is operative under the variable condition that occur in the field.

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