

Bacteria in the Leaf Ecosystem with Emphasis on *Pseudomonas syringae*—a Pathogen, Ice Nucleus, and Epiphyte

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INTRODUCTION

Plants cover a significant proportion of the global land area. Each plant produces many leaves. Each leaf, in turn, may be inhabited by a qualitatively and quantitatively diverse assemblage of microorganisms, including fungi, yeasts, bacteria, and bacteriophages (cf. 158, 229, 249). Colonization of leaves by microbes is not limited to terrestrial plants but includes aquatic plants as well (90). When you view a tree (or any plant), think of it as a support system for 1,000 leaves. Then think of each of these leaves as a habitat for 1 to 10 million bacteria. Then multiply this by the almost infinite number of individual trees and other plants present on our planet, and the diversity of habitats that these leaves provide leads to an appreciation of the enormity and importance of leaf-microbe ecosystems.

In 1961, J. Ruinen published a paper entitled “The Phyllosphere—an Ecologically Neglected Milieu” (249). Up to that time, interest in leaves and their microbial inhabitants was largely centered on diseased leaves and the microbes that caused the various foliar diseases. Phytopathogenic microbes

and the economic impact of diseases on crop production continue to provide a major motivation for research on the phyllosphere as an ecosystem. Interest in leaves as habitats for microorganisms, not merely the causal agents of disease, has grown since the midcentury. Indeed, Ruinen’s research focused on the possible colonization of leaves of plants in the tropics (Indonesia and Surinam) by *Beijerinckia* and other nitrogen-fixing bacteria. Since then, a relatively small but devoted band of scientists has worked diligently to explore the microbiology of leaves. At 5-year intervals, beginning in 1970, international symposia have been organized to provide a forum for discussions on phyllosphere microbiology (1970, Newcastle-upon Tyne, England; 1975, Leeds, England; 1980, Aberdeen, Scotland; 1985, Wageningen, The Netherlands, 1990, Madison, Wisconsin; 1995, Bandol, France; 2000, Berkeley, California). The proceedings of these conferences summarize the progress made in understanding the nature of leaf habitats, the microbes that colonize leaves, and interactions among microbes and between microbes and plants (8, 26, 60, 78, 209, 229). In this review, we focus on the bacterial component of leaf ecosystems.

Although the phyllosphere may no longer be a completely neglected milieu, it continues to be a source of wonderful biological questions and undoubtedly will continue to hold many interesting surprises for future investigators. Not only

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are these communities of bacteria interesting in and of themselves, they are connected directly through their habitats to the reduction of most of the carbon upon which nearly all other organisms depend. Some of these bacteria are known pathogens, destroying or diminishing the photosynthetic output of their leaf habitats. Others are thought to be beneficial. The nature of the interactions, if any, of the majority of leaf-inhabiting bacteria with the leaves that they inhabit remains unknown, an indication of how little is really known about these bacteria.

Before one can integrate the parts of a puzzle into a whole, one must first find the parts and decide which puzzle they belong to. Our interest is the interactions of populations of bacteria with populations of leaves under "real world" field conditions. Factors that affect these interactions include plant and bacterial genetics, weather and other aspects of the physical environment, and plant phenology. Estimates of sizes of such populations are influenced by sampling and bacteriological and statistical methods. The synthesis summarized at the end of the review includes pieces drawn from these several areas. Although the synthesis is an attempt to create a unified picture of the entire puzzle, it still lacks much more than it contains.

We begin this review with a brief general description of the leaf as a habitat for bacteria and what is known about the types of bacteria that colonize leaves. In the remainder of the review we focus on *Pseudomonas syringae*, a species that participates in phyllosphere bacterial communities as a pathogen, ice nucleus, and epiphyte. Among the diversity of microbes that colonize leaves, none has received wider attention than *P. syringae*, as it gained notoriety for being the first recombinant organism (Ice⁻ *P. syringae*) to be deliberately introduced into the environment. Our intent is to use this bacterium to illustrate what has been and can be learned about plant-bacterium interactions by integrating knowledge from population studies in the field and molecular biology studies conducted in the laboratory.

SETTING THE STAGE

General Characteristics of Leaf Habitats

The first piece of the puzzle is the habitat itself. If we allow a rather gross anatomical simplification, leaves can be viewed as consisting of three general components—the epidermis, mesophyll, and veins or vascular bundles (Fig. 1A). The bacteria that are central to this review are found (or thought to be found—more about that later) on the surface of the epidermis and in the intercellular spaces (apoplast) of the mesophyll but not internal to plant cells. Bacteria pathogenic to leaves are thought to occupy the apoplast more frequently than commensal organisms, while both occupy the surface of the epidermis. Bacteria found within the vascular bundles are said to be endophytic and, regardless of their importance, are ignored in this review. Most of the research and interest in leaves as habitats for bacteria have focused on the nature of the leaf surface and its dwellers. This is reflected in terms such as phylloplane to refer specifically to the surface of leaves and epiphytes or epiphytic bacteria to set apart bacteria that are found on the leaf surface from those that are in the apoplast (Fig. 2). The term phyllosphere has been used to describe the environment of leaf surfaces. We interpret environment as including the zone below the surface as well (i.e., the entire leaf). Where appropriate, we use the term phyllosphere interchangeably with leaf associated to refer to bacterial populations in and on leaves.

Certain features of leaf surface topography set these habitats apart from others and evoke intriguing questions as to how bacteria have adapted to life at the interface between the leaf and the atmosphere. The cuticle layer coating the epidermis separates the leaf proper from the surrounding air and functions to protect and waterproof the plant surface (144). The layer, composed of cutin (mainly esterified hydroxy fatty acids) and waxes, forms a lipophilic barrier with low permeability. Dotting the surface landscape are stomates, trichomes, and other leaf surface appendages. All of these structures together with the surface waxes provide a topography that, scaled to the size of a bacterium, is far from smooth and uniform and may be imagined as a jumbled matrix of peaks, valleys, caves, and plains for bacterial colonization (Fig. 1B, 1C, and 2A).

In the past few decades there has been much interest in life in extreme environments, such as hydrothermal vents, acid seepage from mines, and similarly uninviting habitats. That bacteria colonize such environments is not at all surprising to us. After all, they flourish on leaf surfaces! This habitat as well has been frequently regarded as hostile to bacteria. Is the phyllosphere an extreme environment? Compared to a hot spring or hydrothermal vent, the 40 to 55°C sometimes found on leaf surfaces exposed to intense sunlight is relatively benign. Although desiccation of leaf surfaces may provide a stress for bacteria, that stress may be no greater than on exposed skin, very dry soil, or other such habitats. Similarly, the relatively cool 5 to 10°C temperatures and dilute (oligotrophic) substrate concentrations that may be typical of leaves that are wet with dew on a clear night may be relatively benign compared to the cold, dilute conditions of many streams or marine environments. Nor is the UV radiation any more intense on the surface of a leaf than in shallow, clear water. What is unique, or nearly so, to the leaf habitat is that a bacterium on a leaf may be exposed to all of these conditions during the course of a single day and is normally exposed to most of them every day. If the leaf surface is a harsh environment, then the property that makes it harsh is not the extremes to which it is exposed, but the frequent, repeated, rapid alteration between these very different conditions, any one of which may be considered stressful to at least some bacteria.

Thus, an important feature of leaf surfaces that sets the phyllosphere apart from most other types of microbial habitats is that epiphytic bacteria exist in a continuously fluctuating physical environment. Exposed to the atmosphere and the sun, leaf surfaces and, hence, their resident microbes are subjected to changes in aspects of microclimate such as temperature, relative humidity, wind speed, radiation, moisture, and others that occur on time scales of seconds to hours. As moisture levels change, for example, as might occur with the formation of dew during night and its subsequent evaporation during the day, so do substrate concentrations and osmolarity and other measures of water availability and activity. Intense rains may catastrophically perturb the system, as large numbers of bacteria (e.g., 10⁵ CFU per bean leaflet in 15 min [171]) may be removed and deposited on the ground during rainfall.

Leaf habitats are intrinsically ephemeral in nature, surviving only a few weeks for many annual plants in the temperate zone to a few years for tropical or evergreen plants. For plants growing in temperate areas where climate changes are distinctively seasonal, leaves of annual plants emerge, develop, and senesce in a period of several weeks. During this period, the physical and biological nature of the habitat is continually changing as leaves expand, mature, and weather with age. With the onset of winter, these microbial habitats are destroyed. What happens to their resident bacterial communities?

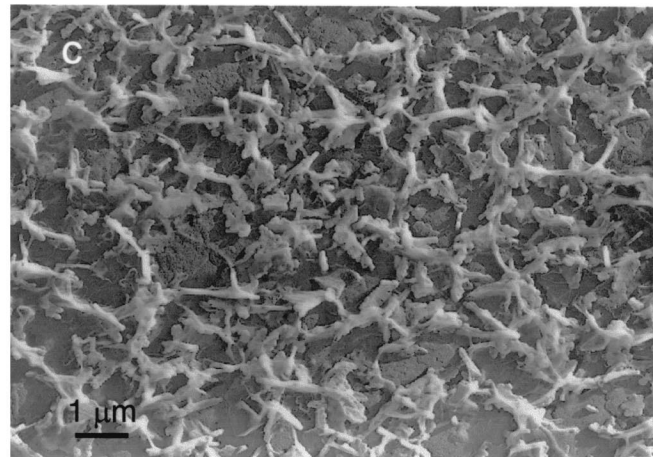
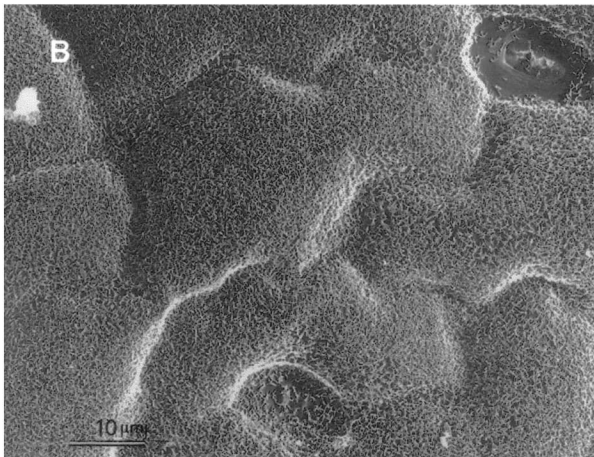
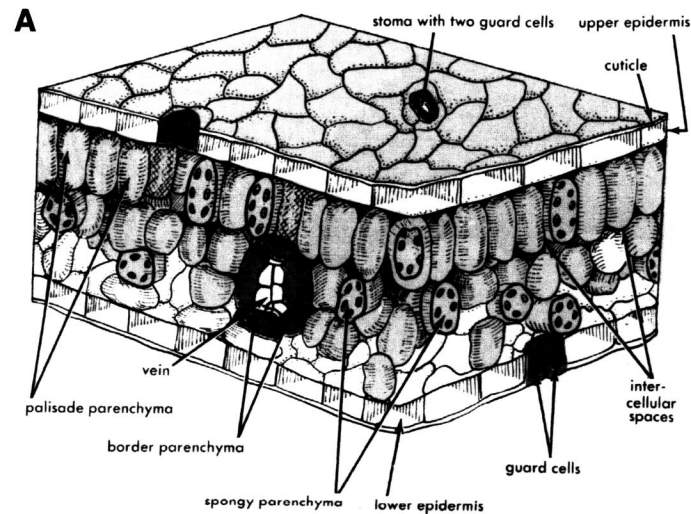


FIG. 1. (A) Three-dimensional schematic of a leaf. Phyllosphere bacteria colonize the surface of the epidermis and the intercellular spaces (apoplast). Reprinted from reference 299 with permission. (B) Low-magnification electron micrograph of the adaxial surface of a pea (*Pisum sativum*) leaf. Reprinted from reference 203 with permission. (C) Electron micrograph of a carbon replica of the adaxial surface of a corn (*Zea mays*) leaf. Note the waxy protuberances. Reprinted from reference 203 with permission.

The Players

Many pieces of the puzzle come in the forms of the kinds of bacteria that inhabit the phyllosphere. A diversity of bacterial species are known to colonize the phyllosphere (Fig. 2 and 3). The specific types and their relative abundances vary with a number of factors related to the plant (e.g., plant species, phenology, and age) and the environment in which the plants are grown (e.g., geographic area and weather conditions within a geographic area) (12, 13, 45, 59, 63, 68, 71, 86, 158, 159, 213, 248, 270, 271, 276, 285, 317). Additionally, what one finds depends on how one looks, and "looking" for phyllosphere bacteria has relied extensively on cultural methods. The extent to which reliance on cultural methods limits the study of bacterial populations and communities remains to be addressed for the phyllosphere (31). The only study to address this issue dealt with one species of bacteria on leaves of growth chamber-grown plants (308). Furthermore, the utility of various ribosomal DNA-based methods (reviewed in reference 6) for studies of bacterial communities in the phyllosphere remains unexplored.

One characteristic of communities of phyllosphere bacteria that has been frequently noted is the abundance (some have termed it preponderance) of pigmented forms (Fig. 3). Indeed, the abundance of chromogenic species common to the phyllosphere (e.g., *Erwinia herbicola* [renamed *Pantoea agglomerans*; however, we will refer to the bacterium by the more familiar name *E. herbicola*], xanthomonads, fluorescent pseudomonads, and pink-pigmented facultative methylotrophs) has led to the yet unresolved speculation that pigment production may confer a selective advantage to bacteria that colonize a habitat which is exposed to solar radiation daily (58, 182, 251, 272). At present there is some effort under way to determine if UV radiation affects the survival or fitness of bacteria in the phyllosphere (273). Once the importance of solar radiation to survival of bacteria adapted to the phyllosphere is better understood, the importance of pigmentation in this process may become better resolved.

Many phyllosphere bacteria are non-spore-forming, gram-negative or gram-positive heterotrophs (13, 68). Some of the bacterial species or individual strains that are well adapted to

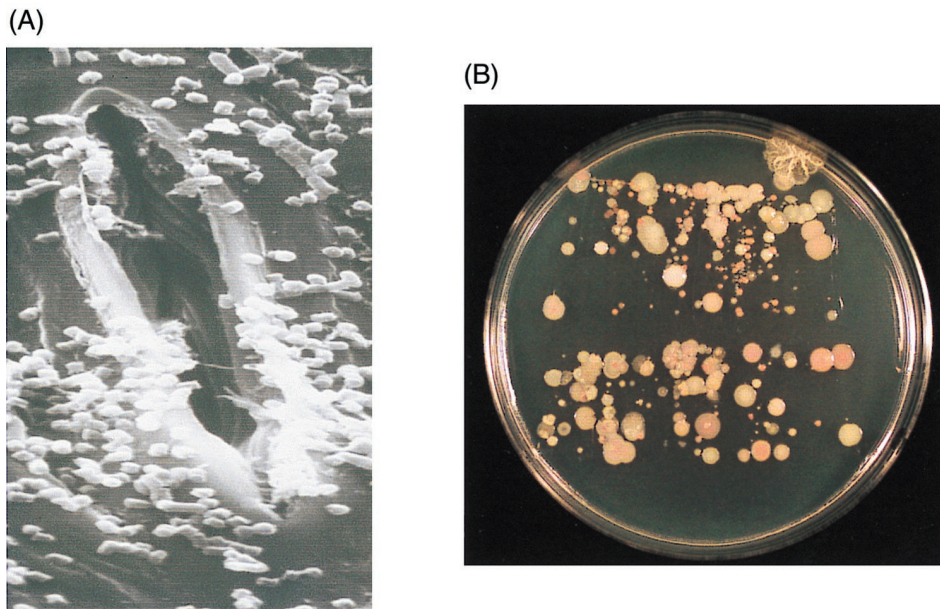


FIG. 2. (A) Scanning electron micrograph of bacteria on the surface of a corn (*Z. mays*) leaf. Photograph taken by J. Lindemann and M. Garment; reprinted from reference 312 with permission. (B) Imprint of a corn leaf. Segments of a field-grown corn leaf were gently pressed onto the surface of King's medium B (146), a nonselective medium.

life on leaf surfaces may be distinct from those commonly found in the soil or rhizosphere (158, 270, 271).

Several studies have examined the distribution of specific groups or species of bacteria of interest across plant species (e.g., ice nucleation-active bacteria [189] and pink-pigmented facultative methylotrophs [45]). In general, such studies have found the same species or groups of bacteria present on a broad range of plants. The conclusion has been drawn that these groups are therefore well adapted to the phyllosphere. Note, however, that such studies do not separate the possibility that these species (or groups) are themselves highly variable and that very different individuals are each well adapted to a few plants from the possibility that most members of these groups are adapted to the phyllosphere of many plants.

There have been fewer attempts to determine bacterial community structure and diversity on a particular plant species (Table 1). Among these are bacteria from leaves of perennial rye (*Lolium perenne*) (59), olive (*Olea europea*) (68, 71), snap bean (*Phaseolus vulgaris*) (210), and sugar beet (*Beta vulgaris*)

(276). Ercolani made two collections of bacteria from olive leaves, roughly 1,700 strains each, during each of two sampling periods spanning several years in two different decades. These collections, which included bacteria present on leaves of different age groups, were characterized phenetically (68–71) (Table 1). Differences in specific types were associated with leaf age and sampling time. Overall, the communities on olive leaves were dominated by strains of *P. syringae* (likely *P. syringae* pv. *savastanoi*, causal agent of olive knot disease). An interesting array of other gram-negative and gram-positive species were identified at lower frequencies. On leaves of perennial rye, the dominant components were identified as strains of *Pseudomonas fluorescens* and *Xanthomonas campestris*, with a number of other minor components (13). On bean, we have observed that the dominant species may change with plant age or with environmental conditions (118). Hence, while the bacterial species composition varies with plant species and other factors, the presence of a few dominant components with a number of species that occur at lower frequencies appears to

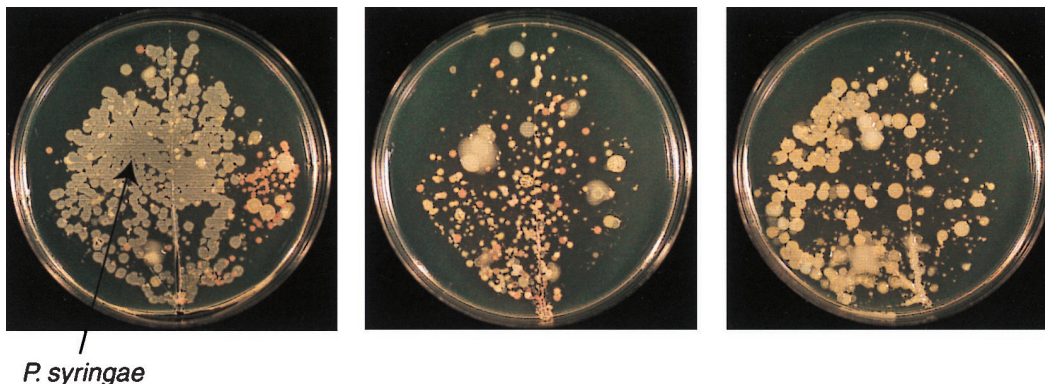


FIG. 3. Qualitative variability in bacterial populations on individual leaflets of field-grown snap bean (*Phaseolus vulgaris*) plants. Leaflets, sampled on the same day, were gently pressed onto King's medium B (146).

TABLE 1. Bacterial communities on leaves of perennial rye (*Lolium perenne*)^a and olive (*Olea europea*)^b

Plant and bacterial component or species	Relative abundance ^c (%)
Perennial rye	
<i>Pseudomonas fluorescens</i>	20.12
<i>Xanthomonas campestris</i>	19.64
"Coryneform bacteria"	8.37
Yellow chromogens.....	4.83
<i>Flexibacter</i> spp.....	4.66
<i>Listeria</i> spp.	4.02
Pink chromogens.....	3.86
<i>Staphylococcus saprophyticus</i>	1.77
Other gram-negative rods	1.61
<i>Klebsiella</i> spp.	0.96
<i>Acinetobacter</i> spp.	0.96
<i>Erwinia herbicola</i>	0.80
<i>Pseudomonas</i> spp.	0.64
<i>Staphylococcus</i> spp.	0.64
<i>Bacillus</i> spp.	0.32
<i>Micrococcus luteus</i>	0.32
Orange chromogens.....	0.32
Unidentified isolates	26.57
Olive	
<i>Pseudomonas syringae</i>	51.0
<i>Xanthomonas campestris</i>	6.7
<i>Erwinia herbicola</i>	6.0
<i>Acetobacter aceti</i>	4.7
<i>Gluconobacter oxydans</i>	4.3
<i>Pseudomonas fluorescens</i>	3.9
<i>Bacillus megaterium</i>	3.8
<i>Leuconostoc mesenteroides</i> subsp. <i>dextranicum</i>	3.1
<i>Lactobacillus plantarum</i>	2.8
<i>Curtobacterium plantarum</i>	2.2
<i>Micrococcus luteus</i>	2.2
<i>Arthrobacter globiformis</i>	1.4
<i>Klebsiella planticola</i>	1.2
<i>Streptococcus faecium</i>	1.2
<i>Clavibacter</i> sp.	0.98
<i>Micrococcus</i> sp.	0.82
<i>Serratia marcescens</i>	0.81
<i>Bacillus subtilis</i>	0.57
<i>Cellulomonas flavigena</i>	0.4
<i>Erwinia</i> sp.	0.37
<i>Zymomonas mobilis</i>	0.3
<i>Bacillus</i> sp.	0.29
<i>Alcaligenes faecalis</i>	0.27
<i>Erwinia carotovora</i>	0.08
<i>Pseudomonas aeruginosa</i>	0.04

^a Adapted from Austin et al. (13).

^b Adapted from Ercolani (71).

^c Total numbers of isolates tested were 621 for perennial rye and 1,701 for olive.

be characteristic of the few phyllosphere bacterial communities characterized thus far. The distribution of bacterial species numbers among leaves thus fits a common pattern observed for other organisms (235).

What ecological roles do phyllosphere bacteria play? Do they have any effect on their habitat, or are they merely adapted to utilize resources otherwise "wasted" by the plant? What is their importance in the global scheme of things?

Bacteria that alter the leaf habitat in obvious ways. We know most about those bacterial species that include strains that visibly modify their leaf habitats. Among these are some of the major groups of plant-pathogenic bacteria (140), pathovars of *P. syringae* and *X. campestris* and *Erwinia* spp., that to various degrees have been found as epiphytes on leaves of their

susceptible host and nonhost plants (cf. 114, 161, 228). Bacterial ice nucleation alters the likelihood of frost injury at temperatures only slightly below 0°C. Some strains of several species that inhabit the phyllosphere are ice nucleation active (INA), including *P. syringae*, *P. fluorescens*, *E. herbicola*, and *X. campestris* pv. *translucens* (reviewed in references 121 and 175). Bacteria that produce plant hormones may also alter leaf and fruit habitats. For example, strains of *E. herbicola* that produce indoleacetic acid cause russetting of pear fruit (194). Habitat modification by pathogenic and ice nucleation-active *P. syringae* will be discussed further in subsequent sections.

Less is known about the ecological functions of the numerous other bacteria that may benefit their leaf habitats.

Nitrogen-fixing bacteria. There are a number of reports on the isolation and identification of free-living nitrogen-fixing bacteria from the phyllosphere of various plants, most frequently from the tropics (2, 30, 139, 156, 214, 223, 248–252, 261, 262, 268, 287). The importance of these bacteria, their frequency or population sizes, and the overall role that they may play in the nitrogen cycle are difficult to assess. Most isolations have been by enrichment culture rather than direct plating, making quantitation difficult. Ruinen reported on the presence of large numbers of nitrogen-fixing bacteria on leaves of a range of plants grown in nitrogen-poor soils in Indonesia and Surinam (248–251). However, her quantitation was by direct microscopic observation of bacteria on leaf surfaces, a method that could not distinguish nitrogen-fixing bacteria from the many other genera present on leaves.

From the work of Jones (139), we do know that measurable amounts of ¹⁵N can be fixed on Douglas fir leaves in situ. Thus, there is no question that nitrogen fixation by foliar microorganisms does occur and may be of some importance for nitrogen cycling in nonfertilized systems. Identification of the actual microorganisms responsible for this nitrogen fixation is clouded, however, because Jones used enrichment culture to isolate such bacteria from his samples.

A large number of papers that report work done in India make it quite clear that nitrogen-fixing bacteria, most commonly *Beijerinckia* and *Azotobacter* spp., can be isolated from many crop plants (214, 222–224, 262–264, 287). Improved plant growth and/or yield of rice and wheat inoculated with strains of *Azotobacter* and *Beijerinckia* spp. has been reported. In addition, bacteria capable of nodulating aerial stems can be found as epiphytes. Adebayo et al. (2) found epiphytic populations of *Azorhizobium caulinodans*, a species that forms stem nodules on *Sesbania rostrata*, at densities of up to 5×10^5 cm⁻² on leaves.

Thus, it is clear that nitrogen-fixing bacteria do occur on leaves, sometimes fairly frequently, and that measurable amounts of dinitrogen can be fixed in situ by at least one plant species, presumably because of the presence of nitrogen-fixing epiphytes. Many important issues such as the identities of the bacteria that are quantitatively important for the nitrogen fixation and the nature of the plant-bacterium interactions remain to be elucidated with regard to this potentially significant aspect of phyllosphere microbiology. Furthermore, there is sufficient data indicative of increases in yield and productivity in response to addition of nitrogen-fixing bacteria to the phyllosphere to suggest that important technological advances in agriculture may result from exploitation of nitrogen-fixing bacteria that inhabit the phyllosphere.

Almost nothing is known of the function in the phyllosphere of those bacteria for which there are no known detrimental or beneficial roles for their plant hosts.

Pink-pigmented facultative methylotrophs (PPFM). PPFMs in the genus *Methylobacterium* are a physiologically interesting

group of bacteria that preferentially utilize substrates lacking carbon-carbon bonds (e.g., methanol and methyl amine) as sources of energy and carbon (92–94, 225). PPFMs (mainly *Methylobacterium mesophilicum*, formerly *Pseudomonas mesophilica*) have been found in large numbers from leaves of a diversity of plant species, including angiosperms (monocots and dicots), gymnosperms, and even lower plants (e.g., bryophytes and ferns) (12, 19, 43–45, 116, 118, 317). In the mid-western and northeastern United States, PPFMs may constitute a significant proportion of the bacterial community on leaves of field-grown plants, depending on plant species and a number of other factors. In other areas (or climates), these bacteria may be less abundant. PPFMs were not detected on leaves of olive plants in southern Italy (68, 71). Although they are not always present in large numbers, PPFMs frequently constituted up to 79% of the heterotrophic bacteria recovered from leaves of white clover (*Trifolium repens*) (45) or more than 90% of the bacteria culturable from snap bean (*Phaseolus vulgaris*) (118). We continue to be impressed with the consistent success (as reflected in their great abundance) of the PPFMs as leaf colonizers. Although they grow slowly in culture relative to, say, *P. syringae* or *E. herbicola*, by the time snap bean pods are ready for harvest PPFMs predictably establish large population sizes (>10⁷ CFU per leaflet), regardless of weather conditions. (*P. syringae*, on the other hand, only does so under favorable weather conditions. More to be said about this in a later section.)

Corpe and Rheem (45) suggested that the success of the PPFMs may be due to their ability to uniquely utilize C₁ compounds (such as methanol) which other, faster-growing heterotrophs are unable to metabolize. Methanol concentrations of leaves measured by Corpe and Rheem (45) and Nemecek-Marshall et al. (215) demonstrate the availability of this C₁ compound on leaf surfaces. It has been suggested that methanol emission from leaves may be a major source of methanol found in the atmosphere (215). Hence, if PPFMs consume methanol as a source of carbon and energy, they may play an important ecological role in the carbon cycle in nature (see also reference 92). Whether this is the case remains to be tested experimentally.

There are several plausible reports that some sort of beneficial interaction with plants may exist for the PPFMs (19, 125, 126). Indeed, plant-PPFM associations were initially recognized when a PPFM was found to contaminate laboratory cultures of the bryophyte *Scapania nemorosa* (liverwort) (19). Because the PPFMs isolated from the liverwort stimulated growth and development of the liverwort and *Streptocarpus prolixus* (flowering plant) in tissue cultures, a positive commensal interaction was proposed (19, 44). That the effect may be due to production of vitamin B₁₂ by the bacteria has been suggested by Basile et al. (18). The hypothesis is based on the associative findings that exogenous application of vitamin B₁₂ to bryophyte cultures stimulated plant growth and a different strain of pink-pigmented methanol-utilizing bacterium produced vitamin B₁₂ (277). Rigorous testing of the hypothesis and elimination of other alternatives remain to be accomplished. Effects of PPFMs on germinating soybean seeds have been suggested to be mediated by cytokinins or other plant growth substances (126). A possible connection between bacterial and plant nitrogen metabolism has also been suggested (125). Thus, findings from laboratory experiments have suggested interesting and unexpected interactions between PPFMs and plants. Additional research is needed to determine if such interactions occur in nature. At this time, what is eminently clear is that PPFMs are nearly ubiquitous on some kinds of plants in some climates. It is also likely that these

bacteria may have some role in modifying their habitat (the plant) or the environment (e.g., via methanol degradation).

PSEUDOMONAS SYRINGAE

The Species: *Pseudomonas syringae* van Hall 1902

P. syringae was initially isolated from a diseased lilac (*Syringa vulgaris* L.) by M. W. Beijerinck in 1899 and subsequently characterized and named by C. J. J. van Hall (318). The species designation is thus linked to the diseased host where the bacterium was first found. Bacteria with similar characteristics have been isolated from diseased tissues of a very large number of other plant species. Although once numbering more than 40 species, all are now classed as the single species *P. syringae* (61). *P. syringae* is easily identified as a gram-negative strict aerobe in the γ subclass of the Proteobacteria which is rod-shaped, with polar flagella, with few exceptions produces fluorescent pigments, is oxidase and arginine dihydrolase negative (phenotypes that distinguish it from most of the other fluorescent pseudomonads), and does not rot potato (which distinguishes it from *Pseudomonas viridiflava*) (61).

The infrasubspecific epithet pathovar is used to distinguish among bacteria within the species that exhibit different pathogenic abilities (64, 319, 320). Nutritional, biochemical, physiological, and nucleic acid-based tests (e.g., DNA hybridization, restriction fragment length polymorphism, and repetitive DNA PCR-based genetic fingerprinting) have also been found useful in determining pathovars of *P. syringae* (49, 54–56, 66, 84, 104, 164, 198, 200, 244, 257, 259, 300, 318, 321). Findings from the various tests are generally in agreement with the groupings made on the basis of host range.

Strains within most of the pathovars exhibit rather narrow host ranges (29). The exception may be pathovar *syringae*, which includes the strain originally isolated from lilac (i.e., the type strain for the species). More than 80 plant species are listed as hosts for strains of *P. syringae* pv. *syringae* (29). From published data, it is not clear whether pv. *syringae* is a repository for strains that may in actuality have quite limited host ranges (36, 164, 198, 247, 253). A single plant species may serve as host for strains within two (or more) different pathovars, one of which is invariably pv. *syringae*. For example, *P. syringae* pv. *tomato* and *P. syringae* pv. *syringae* both cause disease on tomato, bacterial speck and fleck, respectively. Although the symptoms of the diseases are fairly similar (small necrotic lesions), strains within the pathovars are clearly different with respect to a number of phenotypes, including nutritional, biochemical, and serological parameters, phage sensitivity, DNA-based characteristics, and others (48, 54–56, 138).

Thus, the species *P. syringae* has generally evolved to interact with a wide range of plants in most regions of the world. However, within the species, there is a great deal of specialization with respect to plants with which individual strains are likely to interact. Bacteria that cause disease on a plant are usually much better colonists of that plant than those that do not. Further specialization is indicated by the observation that some strains differ with regard to the mechanisms of their interactions with a single plant, as evidenced by the fact that they cause different diseases of the same host.

Must all strains of *P. syringae* be pathogenic? One finds what one looks for. Until the 1970s, plant pathologists were looking for pathogens in diseased tissue. Isolates unable to cause the symptoms of interest would have been discarded. Thus, all known strains of *P. syringae* were pathogens and pathogenicity was one criterion for belonging to the species *P. syringae*. When the association between frost injury to plants and ice-nucleat-

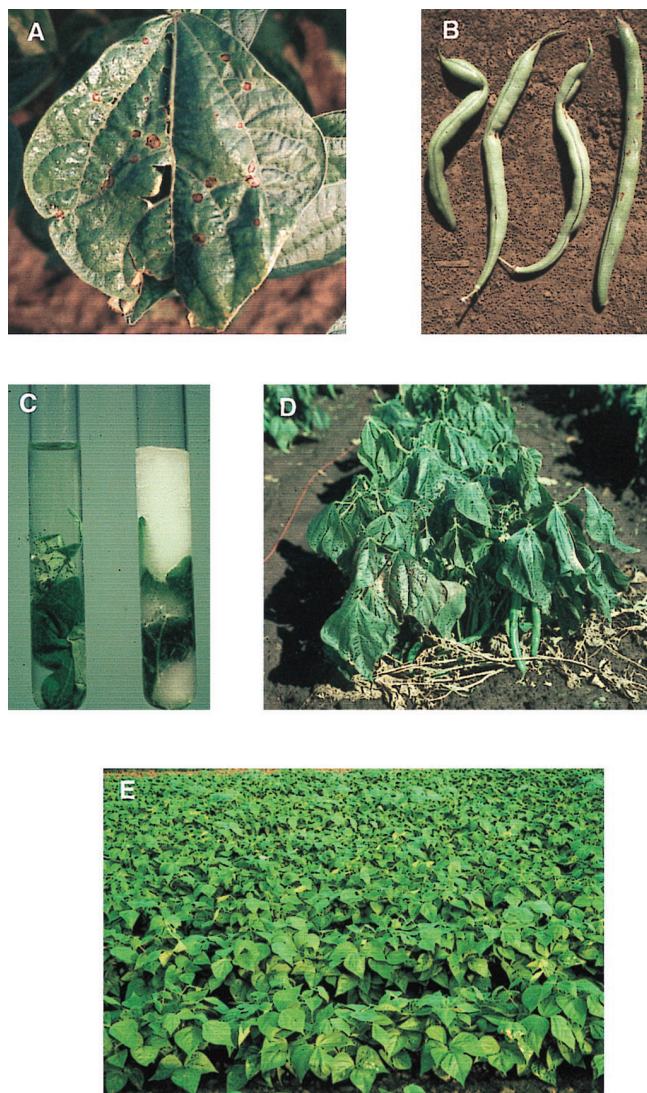


FIG. 4. *P. syringae*—pathogen, ice nucleus, and epiphyte. (A) Foliar and (B) pod symptoms of bacterial brown spot disease of snap bean (*P. vulgaris*) caused by *P. syringae* pv. *syringae*. (C) An ice nucleation event occurred in the test tube on the right due to the large numbers of ice nucleation-active *P. syringae* present on the leaf. (D) Symptoms of frost injury to snap bean plants in the field. (E) Asymptomatic snap bean leaves—habitats for *P. syringae*. Figures B, C, and E are reprinted from reference 111 with the permission of the publisher.

ing bacteria was discovered in the 1970s, searches began for bacteria that were active as ice nuclei (189). The bacteria most frequently found to be ice nucleation active, including the most efficient ice nuclei, had characteristics that placed them in the species *P. syringae* (Fig. 4). However, for some of these strains, plants could not be found on which they would cause disease. The question of whether it was possible that some strains of *P. syringae* might not cause disease at all became one of several important issues in the debate about the safety of deliberate environmental introduction of recombinant organisms. Among the many concerns surrounding the field tests of recombinant Ice⁻ deletion mutants of *P. syringae* (the first deliberate release into the environment of a recombinant organism) was the pathogenic potential of these bacteria. However, the parent strains of *P. syringae* from which the mutants were derived were initially isolated from leaf surfaces of healthy potato (strain

TLP2), citrus (Cit7) and strawberry (S203) (169, 177). Pathogenicity tests were conducted on 30 to 40 different plant species or cultivars, none of which could be identified as a susceptible host for either the parent or Ice⁻ mutant strains. One could argue that plant species X was not tested. After all, is not *P. syringae* a plant pathogen? One could additionally argue that injection of plants grown in growth chambers or greenhouses may not always be a good indicator of the pathogenic potential of a strain, as plant reactions may not resemble those encountered in the field and hence be difficult to interpret. To add fuel to the fire, plant pathologists have long accepted the finding of Klement (153) that the rapid necrotic reaction that develops following inoculation of a nonhost (e.g., tobacco) with large doses of a strain of *P. syringae*, referred to as the hypersensitive reaction (HR), is indicative of its pathogenic potential. Hence, a strain that causes the HR on tobacco must be a pathogen of some other plant species. The parent Ice⁺ and mutant Ice⁻ strains caused the HR when infiltrated into tobacco. On the other hand, if we were not so entrenched with the model that *P. syringae* is a plant pathogen and hence every strain must be pathogenic on some plant species, the results of Lindow (177) and Lindemann and Suslow (169) would not be so difficult to accept. Once again the dogma seemed in conflict with the data. Today many of us accept the concept that *P. syringae* has evolved to live in association with leaves, and incidentally, some (many?) strains cause lesions on some plants.

P. SYRINGAE AND BACTERIAL ICE NUCLEATION

Discovery

Something associated with dead leaves—in one case derived from ground-up dry leaves, in the other from decaying leaf litter—became *the* common focal point for two groups, each pursuing completely different lines of investigation in disciplines as widely separated as atmospheric sciences and plant pathology (282). G. Vali, R. Schnell, and colleagues at the University of Wyoming searched for biogenic sources of ice nuclei that play a role in precipitation processes. S. E. Lindow, D. C. Army, and C. D. Upper at the University of Wisconsin-Madison sought to understand how dried corn leaf powder affected the susceptibility of corn to frost injury. Their paths crossed with the isolation and identification of *P. syringae* from their respective leaf preparations and the discovery of its ability to nucleate supercooled water to form ice (9, 201). The story of the discovery of bacterial ice nucleation as narrated by Upper and Vali (282) is not only entertaining but informative and insightful in depicting the process of scientific inquiry—the delight in finding the unexpected; the frustration in overcoming accepted dogmas; and the skepticism toward accepting the unexpected. The discovery of biological ice nucleation opened new avenues of research ranging from such seemingly unrelated topics as winter survival of insects, snow making, and food processing (163). There are several excellent reviews on bacterial ice nucleation (175, 178, 294, 296, 297, 313, 314, 316). We include here only a few aspects deemed relevant to our overall goal of generating interest in the leaf ecosystem and its bacterial inhabitants.

Ice Nucleation-Active Bacteria, Genes, and Proteins

The ability of bacteria to nucleate supercooled water to form ice is uniquely limited to *P. syringae* and a handful of other bacterial species, many of which dwell in the phyllosphere. (Of course, ice-nucleating bacteria were first found associated with leaves, and this is where people have looked. Searches may not

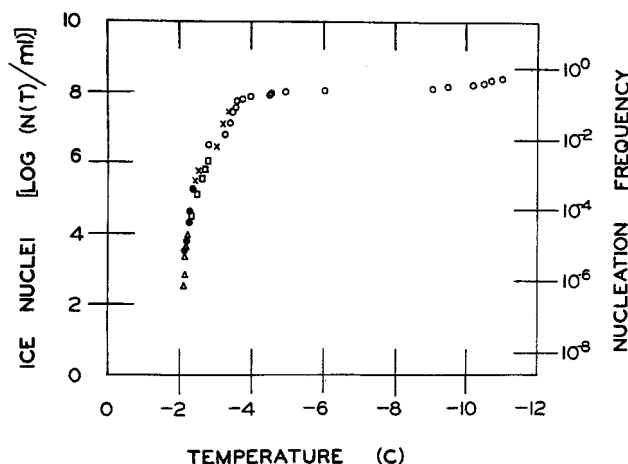


FIG. 5. Ice nucleation spectrum of a strain of *P. syringae*. Cells of *P. syringae* were suspended in phosphate buffer (0.1 M, pH 7.0) to ca. 10^8 CFU/ml. Tenfold serial dilutions were prepared from this suspension. Droplets from the original and each diluted suspension were placed on an aluminum block. The block was cooled, and the temperatures at which droplets froze were recorded. Each symbol represents determinations from one of the five suspensions. The concentration of ice nuclei N at temperature T [$N(T)$] in the suspension was calculated by the method of Vali (283). Nucleation frequency is the ratio of the number of ice nuclei to bacterial cell density.

have been as extensive in other habitats.) Among these are strains of *E. herbicola*-like, *Erwinia ananas*, *Erwinia uredovora*, *P. fluorescens*, *P. viridiflava*, and *X. campestris* pv. *translucens* (9, 142, 145, 190, 201, 216, 217, 226). Not all strains within these species are ice nucleation active. Indeed, ice nucleation activity has been used as one (among several) trait to distinguish strains among some of the *P. syringae* pathovars (108, 138, 226). For example, strains within pv. *syringae* frequently exhibit the ice phenotype, while none of the strains tested thus far within pv. *tomato* or *morsprunorum* are ice nucleation active. Among strains that are active in ice nucleation, not every cell is active at a given time and temperature (Fig. 5) (9, 175, 195, 201). The fraction of cells within a population that are active as ice nuclei increases with decreasing temperatures below -1°C . That only a few bacterial species are ice nucleation active; that not all members within such species exhibit the phenotype; and the stochastic nature of the activity within strains that exhibit the phenotype have raised intriguing questions regarding the evolution and molecular basis for bacterial ice nucleation. Sequence analysis and biochemical characterization of ice genes and proteins have suggested some answers to these questions (292, 295, 314, 316).

Orser et al. (219, 220) were the first to demonstrate that the ice phenotype could be transferred to *Escherichia coli* by expression of DNA clones from the genome of *P. syringae* and *E. herbicola*. Since then, *ice* (or *ina*, for ice nucleation activity) genes have been cloned and sequenced from strains of *P. syringae* (96), *P. fluorescens* (291), *E. herbicola* (290), *E. ananas* (1), and *X. campestris* pv. *translucens* (325). In all cases, the genes contain a single open reading frame (ca. 3,600 to 4,000 bp) with a large central region consisting of a hierarchy of repeated motifs of 24, 48, and 144 nucleotides in length flanked by unique 5' and 3' sequences. The significance of the ordered internal repeats in the function of ice proteins as templates for ice formation has been noted (96, 291, 293). Results of a wide range of experimental and theoretical approaches suggest that ice proteins assemble to form aggregates of various sizes in association with the outer membrane of bacterial cells (32, 42,

52, 74, 91, 95, 141, 155, 196, 212, 267, 280, 290–294, 297, 298, 313–315). The number of protein monomers that are assembled in a proper conformation at any given time may account for the various temperatures at which individual cells are active in ice nucleation. The larger the aggregate, the more efficient the ice nucleus. For an excellent review on ice nucleation theory and measurement, see reference 283. For models of bacterial ice nuclei, see references 141 and 295.

It is curious that only a proportion of the strains within a bacterial species exhibit the ice phenotype. Edwards et al. (65) found a close correlation between the Ice^+ phenotype and presence of an *ice* gene as detected by Southern hybridization using an 800-bp fragment from the internal repetitive region of the *inaX* gene from *X. campestris* pv. *translucens*. The probe hybridized to genomic digests from one of five *E. herbicola* strains, 9 of 26 *P. syringae* strains, one of two *P. fluorescens* strains, and 8 of 16 *X. campestris* strains. With the exception of two strains (one each of *P. syringae* and *X. campestris*), the strains that exhibited an Ice^- phenotype did not hybridize to the probe. The two exceptions apparently contained the *ice* gene but did not express the Ice phenotype under the conditions tested. Thus, the gene was apparently missing, not just not expressed, in most strains that did not exhibit the Ice phenotype. Comparative analyses of the sequences of *ice* genes and proteins, including examination of codon usages, have led to the thought that *ice* genes present today diverged from a common ancestral gene. Wolber (316) suggested that the bacterial species known to contain members that are ice nucleation active acquired the gene via horizontal transfer, perhaps via conjugation between members of "epiphytic communities." It might be interesting to compare the divergence of *ice* genes with those of other genes within ice-nucleating bacterial species.

Ice Nucleation-Active Bacteria and Frost Injury

Although we do not have clear answers as to what selective advantage(s) is conferred upon bacteria that express the Ice phenotype, it is clear that the presence of these bacteria on leaf surfaces can alter (i.e., destroy) leaf habitats at subzero temperatures (9, 174, 175, 188, 191, 195). Frost-sensitive plants are injured when ice forms within plant tissues. Without ice, such injury does not occur. In the absence of heterogeneous ice nuclei, water associated with leaves will supercool. Supercooling in the temperature range of 0 to roughly -5°C is primarily limited by the presence of INA bacteria. Below -5°C , other heterogeneous ice nuclei, including those produced by plants themselves, probably also limit supercooling. Thus, INA bacteria are responsible for ice formation, and hence injury to plants, mainly in the range from 0 to -5°C . Because most cells within a population of INA bacteria are not active at a given temperature and time, the larger the bacterial population, the greater the likelihood that one or more of the cells will be active at relatively warm temperatures (e.g., -2°C). Hence, the amount of frost injury sustained at a given temperature is a function of the population sizes of INA bacteria in the phyllosphere. Once the quantitative relationship between numbers of INA bacteria and frost injury was known, it was apparent that the hazard of frost injury to sensitive crops could be diminished by decreasing population sizes of INA bacteria (174–176).

S. E. Lindow and colleagues demonstrated that population sizes of INA bacteria could be reduced sufficiently to achieve measurable decreases in frost injury by application of naturally occurring and chemically induced Ice^- strains of *P. syringae*, *P. fluorescens*, and *E. herbicola* (174, 176, 178, 184, 192, 193). Ice^-

strains were effective in preventing or minimizing colonization of plants by INA bacteria but not in eliminating established populations of the target microbes. Establishment of relatively large population sizes of the antagonists on leaf surfaces was required for effective exclusion of INA bacteria from leaf surfaces. Hence, manipulation of INA bacterial population sizes by application of non-INA strains is likely mediated by preemptive or competitive exclusion rather than displacement mechanisms (170, 178–180, 184). Competition for limiting resources on leaf surfaces (e.g., nutrients and space) was suggested as a possible mechanism by which established Ice⁻ bacteria may prevent buildup of incoming Ice⁺ strains (309, 310). Because very similar strains of bacteria are involved in such exclusion, any density-dependent mechanism regulating population size is consistent with the available data. Thus, the term competition, in this context, appears to include a broad range of mechanisms, some of which may not normally be thought of as competition. Such a mechanism would imply that antagonist strains with ecological habitat requirements similar or identical to that of the target strains may be more effective in excluding the latter than antagonist strains that are not as closely related. This line of reasoning led to construction of recombinant Ice⁻ strains of *P. syringae* and *P. fluorescens* by deleting a roughly 1- to 1.5-kb fragment (depending on construct) of the *ice* gene, followed by marker exchange of the mutated gene into the genome of the recipient strain (cf. 184, 296). These strains became the first recombinant microbes deliberately released into the environment (181, 184, 197, 296). With the exception of the Ice phenotype, the mutants were similar to their respective wild-type strains in all tests, including lack of pathogenic ability, relative ability to colonize and survive in association with plants in controlled environments, survival in soil, survival following freezing and thawing, and many other traits. Indeed, because of regulatory requirements for these first releases, no *P. syringae* strain has been as extensively and intensively characterized as the Ice⁻ mutants and their respective wild-type strains (169, 181, 197).

After an arduous path through a maze of regulatory, political, and societal obstacles spanning more than 5 years, approval was granted to Lindow and researchers at DNA Plant Technology (formerly Advanced Genetic Sciences, Oakland, Calif.) to field test the Ice⁻ mutants on potato and strawberry plants (181, 184). When the *P. syringae* Ice⁻ mutants and their respective Ice⁺ parental strains were coinoculated onto potato plants in the field, population sizes of the parental strains were reduced over 300-fold (181, 184). Population sizes of naturally occurring (i.e., indigenous) Ice⁺ *P. syringae* were reduced about 50-fold on plants inoculated with the mutants compared to untreated controls. The larger reduction in population size of the Ice⁺ parental strains than of naturally occurring Ice⁺ *P. syringae* by the Ice⁻ mutants is consistent with some degree of specificity in interactions among *P. syringae* strains on leaves. However, the efficacy of the Ice⁻ mutants was similar to that found with some naturally occurring non-INA strains. The reduced population sizes of Ice⁺ bacteria treated with the Ice⁻ strains correlated with reduced amounts of frost injury (ca. 70 to 80%) to potato plants during a natural radiative frost event.

None of the environmental disasters that were suggested prior to the deliberate release of recombinant Ice⁻ bacteria have become reality. This is not surprising given that the experiments were soundly based on what was known about the ecology of *P. syringae* and phyllosphere bacterial population dynamics at the time. The monumental efforts of Lindow and scientists at DNA Plant Technology allowed other scientists, including us, to utilize the power of molecular tools to address fundamental ecological questions on interactions of bacteria

with plants as they occur in the real world. In addition, a commercial preparation of a mixture of naturally occurring Ice⁻ strains is now available for control of frost injury on some crops (184).

EPiphytic Populations of *P. SYRINGAE*

Discovery

For the first half of the century, *P. syringae* was isolated from diseased tissues of many plant species. Its role as a pathogen was firmly established. During this time, lesions were generally viewed as a primary source of inoculum for disease development. Bacteria were thought to move from lesions to susceptible tissue, grow in the intercellular spaces, and cause disease. Growth of the pathogen in other places was not considered important to the biology of the bacteria.

The “birth” of much of our current understanding of the epidemiology of foliar bacterial diseases and interest in leaves as habitats for bacteria can be traced to the seminal findings of J. E. Crosse (East Malling Research Station, U.K.) published in 1959 (46). Among several key findings was the revelation that populations of pathogenic *P. syringae* pv. morsprunorum, the causal agent of bacterial canker and leaf spot of stonefruit trees, could be recovered in large numbers from the surfaces of asymptomatic cherry leaves. Moreover, Crosse suggested that these surface or epiphytic populations of the pathogen and not populations in lesions on cherry leaves, as thought at the time, were the likely main source of inoculum for the infection of stems and branches leading to the development of canker infection. Crosse’s pioneering work was subsequently generalized by C. Leben in the United States into a conceptual framework for the role of epiphytic populations in the epidemiology of diseases caused not only by pathovars of *P. syringae* but also by other phytopathogenic bacteria, most notably pathovars of *X. campestris* (161). The careful work of Crosse and advocacy of Leben gradually brought the possibility that these bacteria may not always reside in lesions to the consciousness of phyto-bacteriologists. Although the dogma that phytopathogenic bacteria are confined to lesions was gradually abandoned, the role of such bacteria growing in association with healthy tissues was not well understood. However, sufficient interest was generated in these so called epiphytic populations of phytopathogenic bacteria that much of the research in phyto-bacterial epidemiology during the 1960s and 1970s was centered on searching for and successfully finding pathogen populations on healthy leaves of host and nonhost plants (cf. 114). The role of *P. syringae* in the phyllosphere was extended to that of an epiphyte as well as pathogen. Because old ideas seldom die without a battle, however, there was substantial support for the idea that these bacteria resided in “latent lesions.” If the function of pathogens is to cause disease, they must be invading tissues and surviving or growing there until conditions that favor lesion development occur. Note the importance in the above argument of the assumption that the primary role of these bacteria is to cause disease. As we will describe below, we still do not fully understand the importance of the ability to cause disease to the overall fitness of *P. syringae*.

Quantitation of Epiphytic Bacterial Population Sizes

In his paper describing “epiphytic” populations of *P. syringae* pv. morsprunorum, Crosse described a method to quantitate leaf surface population sizes of the pathogen (46). Bacteria were removed from cherry leaves by vigorous shaking of leaf samples (2 min every half hour for up to 4 h) in sterile water

(with or without a surfactant) followed by dilution plating of the washings. With some modifications, his basic leaf-washing method became a standard way of enumerating leaf surface bacterial population sizes. Others have since used leaf sonication to remove bacteria from leaves (reviewed in reference 136). Thus, measurement of epiphytic bacterial population sizes has been based on the ease with which culturable bacteria can be removed from leaves by either washing or sonication. While we conceptualize epiphytic bacteria as those that colonize the surfaces of leaves, what we actually measure are culturable bacteria that can be removed by methods such as leaf washing or sonication. The possibility that it may be more difficult to remove bacteria from some parts of the surface structures of leaves than from others and the possibility that either washing or sonication may remove some bacteria from the apoplast (i.e., intercellular spaces) demonstrate the practical difficulties encountered in enumerating "epiphytic" bacterial population sizes. Qualitatively, these bacteria clearly reside within and on leaves. This has been established microscopically. To this day, however, satisfactory methods have not been developed that allow facile quantitative separation of "epiphytic" from "apoplastic" populations of these bacteria. Thus, although it is likely that the bulk of bacteria in leaf washings or sonicates were on the "surface" (i.e., epiphytic), it is also possible that some bacteria in "protected sites" on the epidermal layer were not so readily removed or that some bacteria from the apoplast were. An alternative means to measure numbers of leaf-associated bacteria is to homogenize the leaf and plate from the homogenate. This method provides no information regarding where the bacteria were but does allow somewhat better recovery of bacteria from leaves. We have used homogenization as the method of choice for many years and refer to the bacterial populations enumerated as phyllosphere or leaf-associated bacteria.

Attempts have been made to estimate the proportion of bacteria that are internal, as opposed to on the surface, using various sterilants and UV irradiation of leaves (16, 143, 227, 258, 275, 307; unpublished data). In growth chamber assays, more than 99% of the bacteria (*P. syringae* and PPFMs) that were spray inoculated onto leaves immediately before treatment were killed when hydrogen peroxide was used as the sterilant (unpublished data). Almost none of the bacteria infiltrated into the apoplast before treatment were killed. Thus, this method appears to work quite well for highly artificial controls. When applied to bacteria that have grown in association with leaves in the growth chamber (307) or field (unpublished data), most of the *P. syringae* (75 to >90%) and >99% of the PPFMs were sensitive to the peroxide treatment. Results such as these are consistent with the view that the majority of *P. syringae* and virtually all of the PPFMs are "epiphytic" across a population of healthy leaves.

Enumeration of bacteria by cultural means is dependent on the assumption that the cells of interest (or at least a constant proportion thereof) are recoverable by plating. Wilson and Lindow (308) found that at least 25%, and usually more, of the viable *P. syringae* were recoverable by plating from leaves over a range of conditions in the growth chamber. Thus, estimates of bacterial population size based on culture methods should not be off by more than about a factor of 4. Although fourfold seems like a potentially large error, compared to the normal variability in the field (Fig. 6) and the five to six orders of magnitude across which such population sizes may fluctuate, it is of minor consequence. (The art of measuring population sizes of these bacteria from field samples is such that a factor of 4 is often close to the limit with which differences can be resolved.) Furthermore, the physical environment seemed to

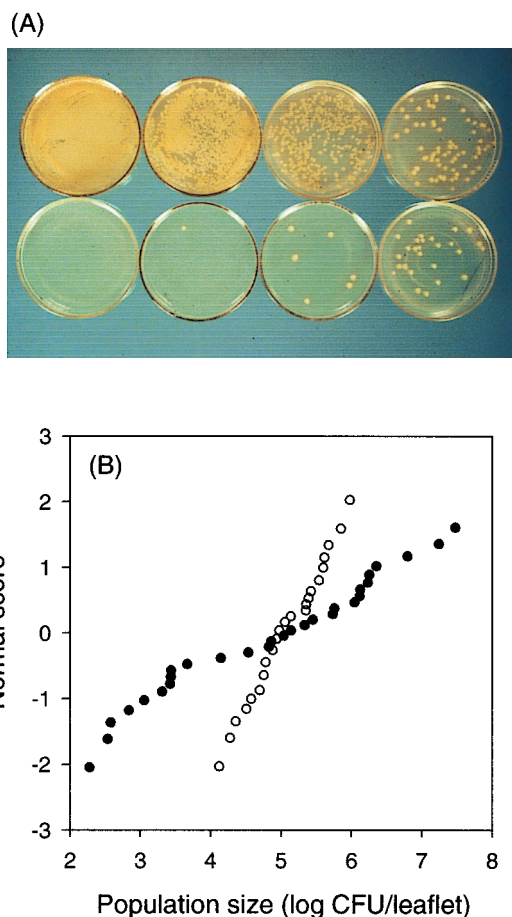


FIG. 6. Quantitative variability in population sizes of *P. syringae* on individual leaves. (A) Each plate represents an equivalent dilution from washings of different individual rye leaves. (B) Lognormal distribution of population sizes of *P. syringae* on two sets of individual bean leaflets. The mean population size for both sets of leaflets is approximately 5.0 log CFU/leaflet. The population variances are 2.5 for set A (●) and 0.23 for set B (○). Reprinted from reference 111 with the permission of the publisher.

have the largest effect on the proportion of cells that were viable but not culturable. In field experiments, samples from all treatments (plots) are normally taken at the same time and hence under the same weather (physical) conditions. Thus, there is some reason to believe that differences in the proportion of viable but nonculturable cells across treatments should be small at any given sampling time. Nonetheless, the possibility that enumeration of phyllosphere bacterial population sizes from field experiments may be influenced by some variable proportion of cells that are viable but do not appear in culture remains an important caveat.

Crosse found great variability among population sizes of *P. syringae* pv. morsprunorum associated with individual leaves (46). Although he chose to combine large numbers of leaves that were washed and dilution plated to achieve some measure of the central tendency of population size, it has since been recognized that much of the information regarding interactions of bacteria with individual leaves resides in the variability about this central tendency (107, 109). Bacterial population sizes often follow (or, more correctly, can be modeled by) the lognormal distribution across populations of leaves (Fig. 6) (109). Less frequently, the related Weibull distribution provides a better "fit" to bacterial population data (134). These

distributions are skewed. Estimation of means by bulking samples (several leaves combined to form a single sample) from such skewed distributions tends to overestimate the mean and underestimate the variability of epiphytic or leaf-associated bacterial population sizes. Variability in bacterial population sizes is present not only among individual leaves but also at other scales, including sites on and in a single leaf (149).

Relationship between Epiphytic Bacterial Population Sizes and Disease

Why are we so concerned about measuring population sizes of *P. syringae* in the phyllosphere? In his original paper, Crosse noted that larger numbers of *P. syringae* pv. morsprunorum were associated with a highly susceptible cultivar of cherry than with a more resistant one (46). Although it is generally accepted that epiphytic populations of *P. syringae* on asymptomatic leaves of susceptible and resistant host plants and on nonhost plants may serve as sources of inoculum for disease, there are few pathogen-host systems for which a quantitative relationship has been found between epiphytic population sizes and subsequent disease. The *P. syringae* pv. *syringae*-snap bean-bacterial brown spot disease is one such system (111, 112, 167, 246). The experimental approaches used by Lindemann et al. (167) and Rouse et al. (246) were similar in the sense that the relationship between epiphytic population sizes of *P. syringae* pv. *syringae* (measured by leaf washings) and amounts of brown spot disease were evaluated in what were essentially dose-response experiments conducted in the field. Different methods, however, were used to establish the necessary range of pathogen doses in experimental field plots. In both studies, pathogen population sizes on sets of individual bean leaflets collected at various times during the growing season were estimated, and information from the lognormal distribution of numbers of bacteria (population means and variances) was taken into account. Lindemann et al. (167) found that mean pathogen population sizes were not predictive of brown spot disease. However, the frequencies with which epiphytic population sizes of *P. syringae* pv. *syringae* were equal to or greater than approximately 10^4 CFU per leaflet on asymptomatic individual bean leaflets were predictive of disease a week later. The probit-lognormal model of Rouse et al. (246) generalized the threshold model by incorporating the notion that the probability of disease can be expressed in terms of the probability of a leaflet having a particular number of bacteria multiplied by the probability of disease occurring on that leaflet given that particular number of bacteria summed over populations of leaves. Fifty percent effective dose values of approximately 1×10^5 to 5×10^5 CFU per leaflet were obtained when the probit-lognormal model was applied to field data. Quantitative models relating bacterial population sizes on individual leaves have successfully predicted disease a short time later. Thus, a quantitative relationship was established between epiphytic population sizes of *P. syringae* pv. *syringae* and the amount of disease that followed. From this finding, in turn, comes the realization that the amount of disease will be determined by the development of large population sizes of the bacteria in association with leaves.

So we arrive at a rather large, paradoxical piece of the puzzle. *P. syringae* has two ways to destroy its own habitat, frost injury and lesion formation. To our knowledge, lesions may provide a place for the bacteria to survive during unfavorable weather conditions but little other advantage. Population sizes of the bacteria are highly variable in lesions but may remain quite large, even after several weeks of dry weather, when population sizes associated with asymptomatic leaves have de-

clined markedly. Although several mechanisms have been proposed that may provide a selective advantage to the bacteria for causing frost injury, none has been demonstrated experimentally. Thus, there is no clear selection for destroying the leaf habitat. For most organisms, habitat destruction is regarded as highly unfavorable. Both of these events, lesion formation and frost injury, become highly likely only when the population sizes of the bacteria are relatively large, that is, when the bacteria have been particularly successful on or in their habitat. The bacteria reward themselves for success by destroying their habitat! Is this not paradoxical? To try to explain this phenomenon, we proposed a model in which lesion formation (and, we should add, frost injury) are unfortunate accidents of overpopulation that benefit neither the bacteria nor the plant (117). The real function of *P. syringae* is to live on healthy leaves. Perhaps it has means to communicate with some plants (we will call them hosts) in ways that improve the habitat for *P. syringae* in some way without causing (much) damage. Only when conditions become unusually favorable and population sizes of the bacteria become too large does the entire system crash, to the detriment of both host and bacteria.

When we first proposed this model a decade ago (117), we did so with tongue in cheek in an attempt to provoke discussion. To our amazement, nothing we have learned in the ensuing decade has provided strong evidence against this model. We reiterate it here to remind the reader that we tend to assign functions to organisms on the basis of those activities in which we are most interested, in this case causation of disease or frost injury. Perhaps we should look harder for those functions with real importance to the bacteria themselves.

Returning to our anthropomorphic interest, the realization that large bacterial population sizes are necessary for either disease or frost injury led to a search for those processes and factors that affect the relative abundance of *P. syringae* in the phyllosphere.

FACTORS THAT AFFECT RELATIVE ABUNDANCE

In this section, we explore what is known or what we think we know about the dynamics of population sizes of *P. syringae* across different time scales. In general, population sizes of *P. syringae* are frequently relatively small on young annual plants or emerging leaves during plant development and may increase to large numbers under suitable conditions (107, 174, 266). On perennials, population sizes of *P. syringae* are generally higher in spring than in summer (67, 68, 98, 174, 202, 243). The time scale over which such changes occur is remarkable. Increases occasionally occur in bursts lasting a day or more, with doubling times of a few hours (116). Infrequently, decreases can occur even more rapidly. For long periods, however, changes are relatively small (107).

At any given time, the number of *P. syringae* (and other bacterial components) present on a given leaf is the sum of immigration, emigration, growth, and death since the emergence of that leaf. Factors that affect the relative rates with which these population processes occur will, in turn, affect bacterial population sizes. For strains of *P. syringae* that are pathogenic and/or ice nucleation active, bacterial population sizes will, in turn, affect the probability of occurrence of disease or frost injury. The challenge is to determine the relative quantitative contributions of immigration, emigration, growth, and death to bacterial population sizes and identify those factors (biotic or abiotic) that influence these processes. This is a daunting task experimentally due to the complexity of interacting factors that operate in nature and difficulties associated with simultaneous measurements of rates at which each of the

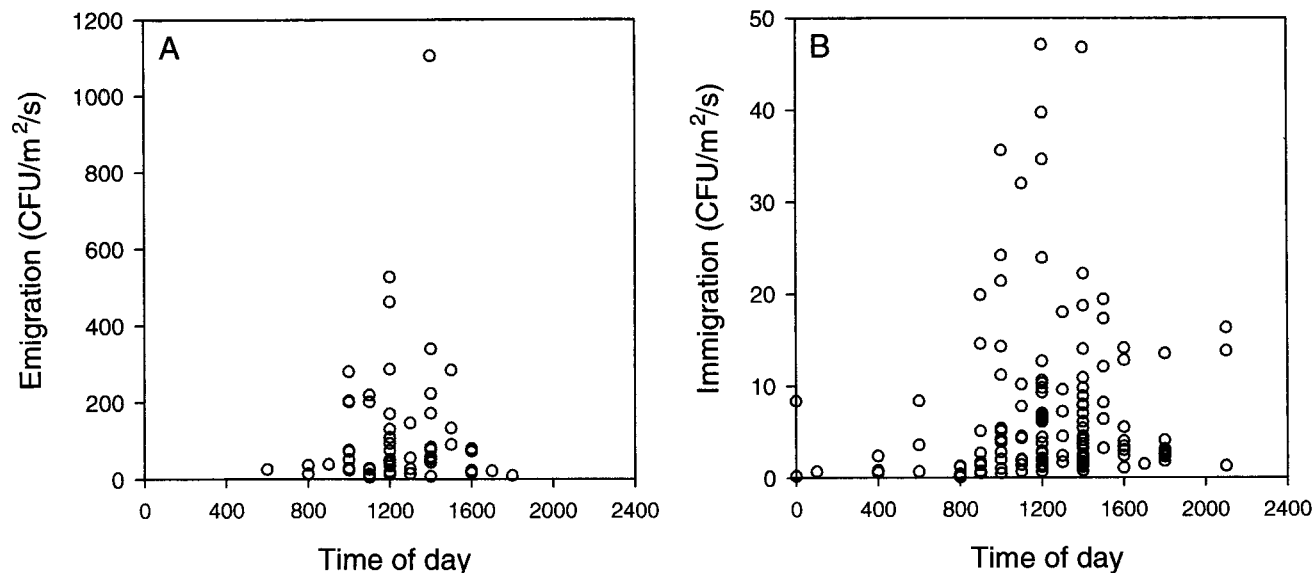


FIG. 7. Immigration and emigration of bacteria to snap bean canopies. (A) Emigration rates are based on measurements of concentrations of air-borne bacteria at canopy height and 1.5 m above the canopy using six-stage Andersen viable samplers (171). (B) Immigration was measured by the deposition of air-borne bacteria onto petri dishes filled to the rim with King's medium B (146). Measurements of immigration and emigration were made over canopies of snap beans during three growing seasons. Data from J. Lindemann; reprinted from reference 118 with the permission of the publisher.

population processes occur in the field (cf. 147). However, some progress has been made, as described below.

Arrival (and Departure)

Bacteria may arrive on leaf surfaces in a number of ways (cf. 288). Many phytopathogenic bacteria, including several pathovars of *P. syringae* and *X. campestris*, may be seed borne (255). Indeed, production of (relatively) pathogen-free seed is an effective way to reduce the hazard from such diseases. Numerous other nonpathogenic phyllosphere bacteria, such as *E. herbicola* and the PPFMs, have been found associated with seeds (126, 286). *P. syringae* grows rapidly on preemergent seedlings and is available to colonize leaves as the plant emerges (80, 110). Presumably, other bacteria behave similarly. Thus, seed-borne pathovars of *P. syringae* are able to flourish in association with the plant below ground as well as above. Although the spermosphere is quite different from the rhizosphere, perhaps it is time to reexamine the issue of whether the separation between phyllosphere- and rhizosphere-associated bacteria is, indeed, as strong as it is thought to be. In field experiments, marked strains of *P. syringae* pv. *syringae* that were inoculated onto bean seeds or foliage were subsequently recovered from the rhizosphere of snap bean and wheat plants (119; unpublished data). The latter was planted as a fall-winter cover crop following a planting of snap beans that was inoculated with a marked strain of *P. syringae* (119). By growing in the spermosphere and in association with preemergent seedlings, *P. syringae* is able to make the transition from seed to leaves, its preferred habitat, and bridge the gap between generations of host plants. Although we have found *P. syringae* in the rhizosphere, we have no evidence that it is able to move from roots to aboveground plant parts. Emigration and immigration of bacteria from one aerial part of a plant to another (such as from buds to leaves) without physical departure of the microbes from plants is also expected but has been less critically demonstrated to occur in the field.

Leaf habitats are unusually open systems. Exposure to the

atmosphere provides ample opportunity for aerial immigration and emigration of phyllosphere bacteria. Indeed, plant canopies were found to be a major source of bacteria in the troposphere, at least during the growing season in those parts of the temperate zone that support extensive plant growth (168). In a study on the effect of cropping patterns on population sizes of *P. syringae* pv. *syringae*, Lindemann et al. (166) found that numbers of the pathogen were larger on leaves of snap bean plants established in experimental plots within a major snap bean-growing area than in plots planted away from the commercial snap bean production area. The seed with which all of the plots were planted was naturally infested with the pathogen. Presumably, *P. syringae* that originated in the large commercial plantings of snap bean dispersed (probably aerially) to the experimental plots. These additional immigrants were sufficiently abundant to influence the population sizes in the experimental plots. Regardless of what the mechanism turns out to be, there is clearly a measurable effect of surrounding vegetation on population sizes of *P. syringae* (166; S. S. Hirano, J. A. Virata, K. A. Lindgren, and C. D. Upper, abstr., *Phytopathology* 87:S42, 1997).

Lindemann and Upper (171) found that although viable bacteria were present in the air at all times of day, significant upward fluxes of bacteria (i.e., emigration) occurred only during the warmest part of sunny days, when leaves were dry and wind speeds exceeded about 1 m s^{-1} . Emigration rates were highly variable and lognormally distributed, with a median value on the order of about $100 \text{ CFU m}^{-2} \text{ s}^{-1}$ (Fig. 7A). On average, this corresponds to a loss of roughly 10^3 CFU per h per bean leaflet with an area of 50 cm^2 . Immigration rates, as measured by the number of bacteria deposited onto the surface of solid medium in petri dishes exposed at canopy height, were also highly variable (Fig. 7B). Most of the time (67% of 130 measurements made over three growing seasons at various times during the day and night), deposition rates were between 1 and $10 \text{ CFU m}^{-2} \text{ s}^{-1}$. This corresponds to roughly 18 to 180 bacterial immigrants arriving per h on a bean leaflet 50 cm^2 in area. From the estimates of Lindemann and Upper (171), we

are left with the conclusion that at least during dry sunny weather, on average, there is a net loss of total culturable bacteria from bean canopies. The net loss was estimated to be over 10^4 CFU per bean leaflet per day during those periods when atmospheric conditions were conducive for dispersal.

On plants such as citrus where little growth of *P. syringae* occurs and population sizes of *P. syringae* are normally quite small, immigration appears to be an important contributor to the numbers of bacteria found on leaves (185, 187). Both bacterial population sizes and immigration rates, measured as rates of deposition on exposed petri dishes, were highest near the edge and decreased with distance away from the edge of a citrus grove (187). Epiphytic bacterial population sizes were only slightly larger than could be explained by total numbers of immigrants summed over several months. On this basis, it was suggested that there may have been no growth of bacteria on citrus leaves—that all of the bacteria on these leaves had grown on nearby plants, moved to the citrus leaves, and accumulated there by deposition (187).

The estimates of Lindemann and Upper (171) were for total culturable bacteria. Rates of immigration and emigration for specific bacterial components and species over snap bean canopies are expected to be smaller than those noted above. In field experiments in which numbers of *P. syringae* were estimated on a series of deposition plates exposed during a continuous 11- or 14-day period, the average number of *P. syringae* arriving on a bean leaf-sized area was about 35 CFU per day (S. S. Hirano, J. A. Virata, K. A. Lindgren, and C. D. Upper, *abstr.*, *Phytopathology* 87:S42, 1997). Such numbers of immigrants are orders of magnitude too small to account for the population sizes of *P. syringae* frequently encountered on leaves of snap beans or other plants that carry relatively large numbers of bacteria in the field. So we are faced with another paradox. Immigration rates are too small to affect population sizes of *P. syringae*, yet strong circumstantial evidence points to the availability of immigrants as very important to relative population sizes of this bacterium on plants such as beans, where the bacteria grow to relatively large population sizes (166).

There is a plausible explanation for how small numbers of immigrants can have a relatively large effect on population sizes of *P. syringae*. The numbers of bacteria associated with very young leaves, as they first begin to expand, are very small. New leaves provide (nearly) empty habitats for bacterial colonization. If the immigration rate of *P. syringae* is low relative to that of other bacteria, then the probability that a given new leaf will receive even one immigrant of that species is very low. Immigrants of some other species will probably arrive first and begin the process of colonization of the leaf. Thus, by the time that a *P. syringae* immigrant arrives, its likelihood of success is smaller than if it had been the first bacterial cell to arrive on the leaf. When the numbers of *P. syringae* available to immigrate to a new leaf are large, then *P. syringae* is more likely to arrive early in the process of colonization of the leaf, when there is ample empty habitat. Hence, leaves subjected to large numbers of immigrants of *P. syringae* are likely to carry larger population sizes of that bacterium.

Immigration and emigration of phyllosphere bacteria also occur during rain. The classical explanation for the air-borne dissemination of foliar bacterial pathogens is that they are carried by the wind in aerosols or ballistic particles generated when raindrops strike diseased plant parts or plants supporting surface bacterial populations (33, 50, 72, 75–77, 288, 289). Thus, outbreaks or spread of bacterial foliar blights have been attributed to dissemination of the pathogen by rainsplash or windblown rain (50, 289) (more to be said about this later).

Although bacteria may immigrate to leaves as they are dispersed by rainsplash, the contribution of these cells to bacterial population sizes is small. This is because during rain, the net flux of bacteria is downward (38, 118, 171). Most of the bacteria that are removed from leaves during rain move downward toward the soil. The large numbers of bacteria collected in rainwater below plant canopies suggest that emigration during rain could account for some of the large, rapid decreases in leaf surface bacterial populations associated with rain events (107, 111, 118, 171).

The role of insects in dissemination of phyllosphere bacteria is not totally clear. For some foliar pathogens, specific insect vectors are thought to be required for dispersal (cf. 288). From the studies of Lindemann et al. (171), we had concluded that in the absence of rain, dispersal occurred mainly during the day, when leaf surfaces are dry. Lindemann frequently observed insects visiting petri plates exposed to measure bacterial deposition. She marked such plates and examined them to determine any association between insect tracks and bacterial colonies. She found none. Due to the absence of bacterial colonies arising from the insect tracks, insects were not given much consideration as vectors of leaf surface bacteria. In more recent field experiments in which deposition plates were exposed “around the clock” for continuous periods of 11 to 14 days, larger numbers of bacteria were present on the plates exposed during the early morning hours, when leaves were wet with dew, than during midday (111; E. M. Groth, S. S. Hirano, L. S. Baker, and C. D. Upper, *abstr.*, *Phytopathology* 85:1188, 1995; Hirano et al., *abstr.*, 1997). The bacterial colonies that arose on the plates were not randomly distributed but rather followed “tracks” of faint footprints left by the insects (Fig. 8). It appears that when leaves are wet, insects traversing the surfaces become contaminated with epiphytic bacteria. Such bacteria, including *P. syringae*, emigrate from and immigrate to leaves as insects flit and fly about. The process appears to be independent of either insect species or bacterial species.

To Grow or Not To Grow

The fate of a newly arrived *P. syringae* in its leaf habitat depends on a number of interacting factors related to the biological, physical, and chemical environments. A factor that plays a dominant role in the initial interaction is plant genotype. Plant species differ in the numbers of *P. syringae* and other bacteria that they normally support in the field. For example, plants such as potato and citrus generally harbor smaller numbers of bacteria than do plants such as almond and snap beans. Populations of bacteria on navel orange leaves appear to be largely accumulations of immigrants (187). It has been suggested that perhaps the thick waxy cuticle layer present on citrus leaves limits the diffusion of nutrients onto the leaf surface, thereby limiting bacterial growth (187). Alternative explanations, of course, remain to be ruled out. Large differences in population sizes of *P. syringae* are common even on leaves of plants of the same species. Breeding for disease-resistant plants has long been a practice to better manage plant diseases. In general, larger population sizes of pathogenic *P. syringae* are established on susceptible cultivars than on more resistant cultivars, even in the absence of disease (14, 47, 51, 206, 207, 269). Little work has addressed the mechanism(s) underlying the effect of host genotype on *P. syringae* population size despite the integral role that such growth plays within the larger question of the basis of disease resistance to *P. syringae*.

Some information regarding the overall role of climate on development of populations of *P. syringae* is available from a number of studies in which samples were taken weekly,

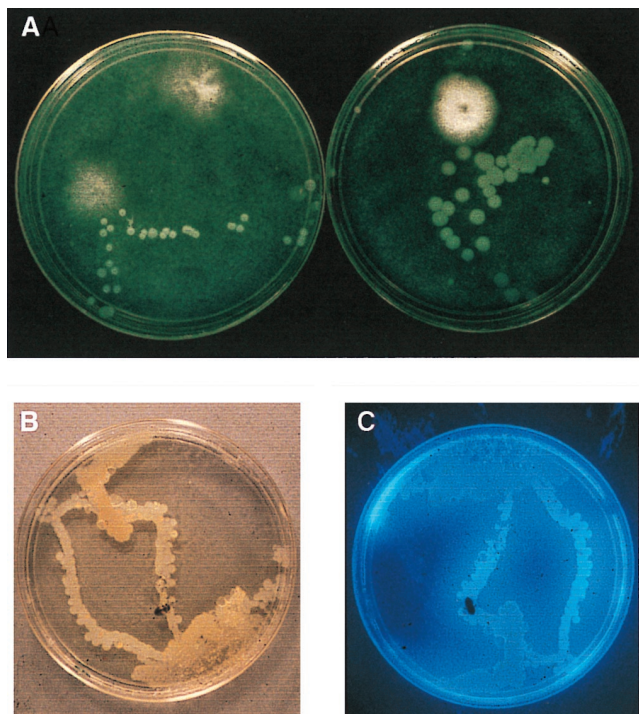


FIG. 8. Insects as dispersal agents of *P. syringae* in the field. (A) Nonrandom distribution of *P. syringae* colonies on a semiselective medium (208) for *P. syringae*. The petri dishes, deployed at canopy height in a bean field, were exposed from 0600 to 0900 h, when leaves were wet with dew. (B and C) An insect (*Glischrochilus quadrisignatus*) was trapped in a sterile empty petri dish exposed in a bean canopy in the early morning. The trapped insect was transferred to a petri dish containing King's medium B (146) and allowed to walk over the surface of the medium. The white colonies in panel B are *P. syringae*. (C) The colonies fluoresced under UV light. Panel B is reprinted from reference 111 with the permission of the publisher.

monthly or even quarterly across one or more seasons (reviewed in references 115, 118, and 120). Generally speaking, net increases in bacterial population sizes were associated with relatively moist conditions (particularly rain and high relative humidity) and relatively cool temperatures. Declining or stable population sizes of *P. syringae* have similarly been associated with dry, hot weather conditions.

The physical conditions experienced by microorganisms on leaf habitats undergo extensive fluctuations each day. Thus, the more interesting question, one that may begin to explain differences between bacteria adapted to the phyllosphere and those better adapted to environments less likely to undergo extensive short-term fluctuations in the physical environment, is how does the weather affect the growth and death of *P. syringae* in the phyllosphere. Sets of individual leaves were sampled from field-grown snap bean plants either 5 or 7 days per week for 6 to 7 weeks (107, 111, 118). The bacterial population sizes that were measured were those of naturally occurring *P. syringae* (the plots were not artificially inoculated) on two cultivars that differ in susceptibility to bacterial brown spot disease. The data set that resulted included changes in bacterial population sizes from 11 plantings of snap beans spanning six different growing seasons. Examples are illustrated in Fig. 9. The effect of host genotype is illustrated by the generally larger population sizes of *P. syringae* on cultivar 'Eagle' than 'Cascade' (cv. 'Eagle' is more susceptible to brown spot disease than 'Cascade') (Fig. 9C). On a season-long basis, population sizes of *P. syringae* and the relative proportion of total cultur-

able bacterial communities attributed to *P. syringae* may differ significantly from one planting to the next (Fig. 9C, D, and E). A comparison of daily changes in *P. syringae* population sizes and weather parameters across all plantings suggested a strong association between the occurrence of rain and the onset of periods of large increases in bacterial numbers. Estimates of immigration rates during either dry or rainy conditions were too small to account for the net increases in *P. syringae* population sizes. Hence, the net increases in numbers of *P. syringae* following rains were due to growth of the bacterium. In two of the plantings, weather conditions were dry and hot. Rainfall was minimal, and population sizes of *P. syringae* were generally below detection levels (Fig. 9E).

Such results only create a positive association between rain and relative abundance of *P. syringae*, which confirmed what others had previously found with less intensive samplings. Although the link between rains and increased population sizes was still just an association, we had added a few more pieces to the puzzle. Increases in population sizes of *P. syringae* associated with rains were due to growth, not immigration. In rain events that led to such bursts of growth, peak rainfall rates met or exceeded approximately 1 mm min^{-1} (105). Population sizes of *P. syringae* were usually lower if measurements were made very soon after rain, presumably due to washoff. Rains of lesser intensity did not trigger growth of *P. syringae*. Population sizes of *P. syringae* tended to remain the same or decline in the absence of intense rains, even though the leaves were wet with dew. Thus, there was no association between leaf wetness per se and bursts of bacterial growth. Furthermore, neither the temperature during the wet period nor the duration of time that leaves remained wet appeared to be an obvious factor in triggering the onset of growth of *P. syringae* on bean leaves.

Inert fiberglass screens were placed over rows of bean plants to absorb the momentum of raindrops but allow the natural volume of water to fall on the leaves without altering its chemical composition or quality (105). In each of three such experiments, population sizes of *P. syringae* increased, as expected, on plants that were exposed to the rains but not on plants under the screens or on plants shielded from the rains by plastic shelters (Fig. 10). The latter treatment provided direct evidence for the positive effect of rain on growth of *P. syringae*. The screen treatment further suggested that the momentum of raindrops plays an important role in triggering the onset of growth of *P. syringae* on bean leaves. The positive effect of rain on the relative abundance of *P. syringae* does not appear to apply to the PPFMs. Population sizes of the PPFMs were larger on plants that were shielded completely or screened from natural rains. Indeed, for the two plantings of snap beans in which *P. syringae* population sizes were at the limit of detection of our plating assay (Fig. 9E), the PPFMs constituted a significant proportion of total culturable bacterial population sizes (Fig. 11). Although both bacterial components are very successful colonists of leaves of snap bean plants grown in Wisconsin, as measured by their relative abundance, they have apparently evolved very different life strategies to achieve success.

The mechanism by which intense rain triggers growth of *P. syringae* remains unknown. Because *P. syringae* grows quite well following misting onto plants in the growth chamber, we lack the negative controls necessary to examine the triggering phenomenon under controlled conditions. This is but one example of how the behavior of *P. syringae* observed in the field differs from that in the growth chamber (281). Caution needs to be exercised in extrapolating findings from controlled environments to the field situation.

Intense rains play a significant role in the relative abundance

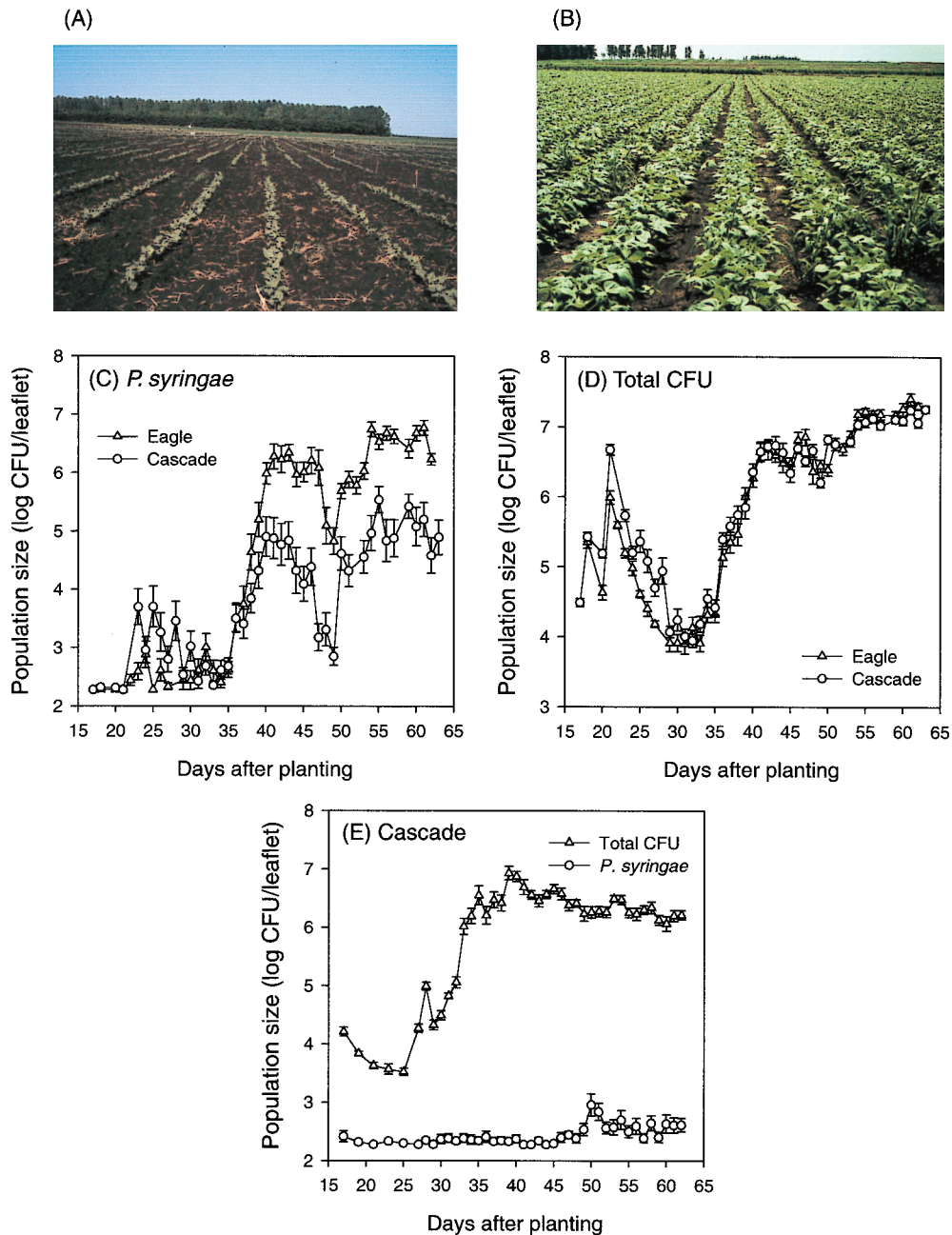


FIG. 9. Population dynamics of *P. syringae* and total culturable bacteria in association with snap bean leaves. (A and B) Plants at about 20 and 60 days after planting, respectively. (C and D) Population sizes of *P. syringae* and total CFU on bean cultivars that differ in susceptibility to brown spot disease. Cultivar 'Eagle' is more susceptible than cv. 'Cascade'. Reprinted from reference 105 with the permission of the publisher. (E) Bacterial population dynamics on cv. 'Cascade' during a growing season with little rain and high temperatures. (C, D, and E) At each sampling time, 30 individual leaflets were collected from the top of the canopy at 0800 h. Each sample was processed individually by dilution plating of leaf homogenates. The data are the means and standard error (SE) for each set of 30 leaflets. Variability in population sizes among the individual leaflets within a set is exemplified by the data shown in Fig. 6B.

of *P. syringae* in the phyllosphere. Such rain is not always required for growth of *P. syringae* in the phyllosphere, however. The bacterium does quite well in some climates and during some seasons when such rains are highly unlikely. Although we suspect that a combination of leaf wetness and extended periods of relatively low temperatures are the likely triggers that replace intense rain in these situations, we have no direct evidence that this is the case. In addition, other parameters of the physical environment (temperature, relative humidity, etc.)

and other factors such as community composition and abundance and interactions among all of the biotic and abiotic factors likely influence the numbers of *P. syringae* at any given time and on any given leaf (70, 71, 85, 135, 148, 210, 218, 309, 310). The system is dynamic and only slightly understood.

The possibility has been raised that availability of nutrients has an effect on numbers of epiphytic bacteria (210, 309, 310). Nutrients in the form of carbohydrates, amino acids, and organic acids have been identified in leachates of leaves (57, 85,

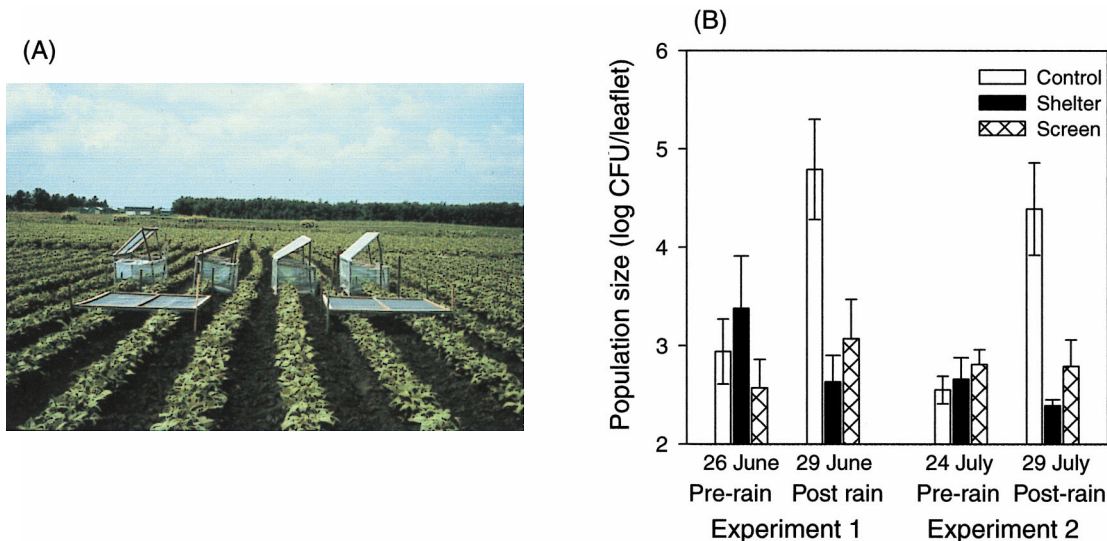


FIG. 10. Effect of raindrop momentum on population sizes of *P. syringae*. (A) Modification of the microclimate with polyethylene shelters and inert fiberglass screens. The shelters were used to shield bean plants from rain when rain was imminent; the screens were used to decrease the momentum of raindrops as they passed through the screens and dripped onto the plants. (B) Phyllosphere population sizes of *P. syringae* on bean plants exposed to natural rains (control), plants shielded from rains (shelter), and plants under screens. Each datum point represents the mean log CFU per sample and SE based on three replicate plots per treatment with 10 individual samples per plot. Reprinted from reference 105 with the permission of the publisher.

278, 279). However, a consideration of the permeability of the cuticle has brought into question the routes by which such compounds may arrive on leaf surfaces (260). At some level, nutrients must be important for these bacteria to colonize leaves. After all, they cannot grow without a source of carbon, nitrogen, phosphorus, other minerals, and energy. The issue is what role, if any, nutrients play in determining or regulating population sizes of *P. syringae* and related bacteria on leaves.

Available evidence suggests that *P. syringae* population sizes can be increased by addition of nutrients to the phyllosphere, either by spraying nutrients onto leaves (210, 309) or by supplying nutrients that might not otherwise be there by use of transgenic plants (311). Changes in bacterial population sizes in response to addition of nutrients to leaves were relatively small compared to normal variations in sizes of their populations in the field (210, 309).

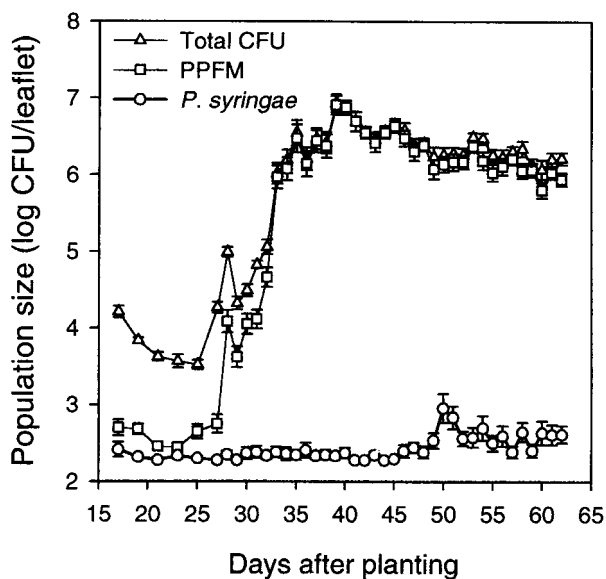


FIG. 11. Population dynamics of PPFMs, *P. syringae*, and total culturable bacteria in association with leaves of field-grown snap bean plants (cultivar Cascade). Under the relatively hot and dry weather conditions that prevailed during the experiment, the PPFMs flourished while *P. syringae* did not. Each datum point represents the mean and SE of bacterial population sizes based on 30 samples, collected at 0800 h each morning, and processed individually by dilution plating of leaf homogenates.

Although the following experiments were done with a strain of *P. fluorescens* (A506) and not *P. syringae*, the findings provide some insight on the role of nutrients in regulating epiphytic bacterial population sizes while simultaneously raising a number of unresolved questions. To test the hypothesis that the availability of sugars on leaves determines population sizes of epiphytic bacteria, Mercier and Lindow examined the relationship between the abundance of sugars on leaves of greenhouse-grown snap bean plants and population sizes of *P. fluorescens* A506 during and after colonization of the leaves (205). Amounts of sugars that could be washed from individual leaves were lognormally distributed, as were population sizes of bacteria recovered from a different set of individual leaves. The extent of variation was similar for the two parameters. Amounts of mono- and disaccharides decreased to about 28 to 37% following bacterial inoculation, compared to amounts present at the time of inoculation. The authors interpreted this decrease as evidence that the bacteria utilized these carbohydrates. These and other arguments were presented as strong evidence that carbohydrates limit population sizes of epiphytic bacteria. However, it is curious that when sugars and bacterial population sizes were followed as a function of time, carbohydrate depletion stopped at about the time that the bacteria entered log phase (ca. 18 h postinoculation), before about 80% of the bacterial growth had occurred. Thus, it appears likely that the bacteria were not utilizing carbohydrates during log phase, in which case it is unlikely that their growth would be limited by depletion of carbohydrates. Alternatively, efflux of carbohydrates to leaf surfaces may have occurred during log-phase growth of Pf A506. (However, this seems an unlikely

scenario, because amounts of carbohydrates were depleted following inoculation with the bacteria.) For carbohydrates to limit growth in this case, efflux would have had to stop at the end of exponential growth. Mercier and Lindow did note that carbohydrates were not exhausted from the leaves but decreased to a steady-state level 25 to 40% of initial amounts. They took this as evidence that the bacteria and this residual carbohydrate must be spatially separated on the leaves. Although this is a plausible explanation, so is the alternative explanation that the bacteria were neither growing on nor limited by carbohydrates on the leaves during mid- to late log phase.

Thus, when all of the work relating nutrient levels to population sizes of *P. syringae* and other bacteria in the phyllosphere is considered together, we must conclude that the relative importance of even this most intuitively obvious of the factors regulating population sizes of bacteria associated with leaves remains obscure.

GENES AND TRAITS THAT CONTRIBUTE TO FITNESS

A question of universal biological interest is what genes and traits (phenotypes) enable an organism to flourish in its habitat. Rhetorically, why do microbes live where they do, be it in an environment considered extreme (e.g., hydrothermal vents) or as commonplace as leaves? Efforts to identify genes and traits that confer fitness on *P. syringae* in the phyllosphere can be largely grouped into two not necessarily mutually exclusive efforts. What genes and traits contribute to the fitness of *P. syringae* as an epiphyte, i.e., to life on leaf surfaces? For strains of *P. syringae* that are pathogenic, to what extent do genes required for pathogenicity or virulence contribute to bacterial fitness in the phyllosphere? The sum of what has been learned from both efforts thus far tells us little more than that this is a necessary direction to follow if we are to fully understand the interactions of *P. syringae* with leaf habitats.

Traits and Genes That Confer Epiphytic Fitness

For a comprehensive treatment of this topic, we refer our readers to the reviews of Lindow (182) and Beattie and Lindow (20, 23, 24). The search for traits that confer epiphytic fitness stems largely from the perspective that the surfaces of leaves provide a rather hostile or harsh place for microbial life. Traits that have been suggested as contributing to the fitness of epiphytic bacteria include pigment production to tolerate UV irradiation, extracellular polysaccharide production to withstand desiccation, and a number of others. In no case has the actual role of these traits in epiphytic fitness been rigorously tested (reviewed in reference 182).

To identify traits that confer epiphytic fitness, Lindow et al. (183) used random transposon mutagenesis of *P. syringae* followed by a screen for mutants with impaired epiphytic growth or survival. It was thought that such an approach may reveal novel (as well as anticipated) traits that contribute to epiphytic fitness. Mutants of wild-type *P. syringae* pv. *syringae* strain B728a (causal agent of bacterial brown spot of snap bean) with altered epiphytic fitness were identified by their diminished population sizes on leaves of snap bean plants subjected to alternating wet-dry conditions. A tube nucleation test, which measures the frequency with which individual leaves carry ice nuclei active at or above -2.5°C , was the basis for a rapid assay to screen for diminished bacterial population sizes relative to the wild type. This is possible because the probability of ice nucleation events occurring at relatively warm temperatures (e.g., -2.5°C) increases with increasing numbers of INA bac-

teria. Of 5,300 mutants screened in this way, 109 were found that caused a smaller proportion of leaves to freeze compared to the parent strain (183, 186). The number was reduced to 82 when 27 were subsequently found to be reduced in ice nucleation activity but not population size, as measured by dilution plating.

The 82 mutants varied in the extent to which population sizes increased or decreased when they were subjected to the wet-dry-wet regimen (186). Nine of the mutants appeared to be similar to the parent strain in the magnitude of the decrease in population sizes during the dry period but differed from B728a in net growth during the wet periods. Most of the mutants, however, were impaired in their ability to survive the "stressful" (dry) conditions compared to the parent strain. Among this set of stress-intolerant mutants, net growth was either less than, equal to, or greater than that of B728a during the wet periods. Hence, characterization of the population dynamics of the large number of mutants relative to the wild type demonstrated the complex nature of a phenotype summarized as epiphytic fitness.

Among the 82 epiphytic fitness mutants, only three were auxotrophs (methionine, tryptophan, and isoleucine/valine). Thus, the frequency of auxotrophs among epiphytic fitness mutants was only about four times that expected of auxotrophs among all mutants. This might suggest that auxotrophy per se is not a major impediment to successful colonization of bean leaves under laboratory conditions. The behavior of two of the auxotrophs (Met and Trp) and two other mutants was examined further in laboratory and field experiments (7, 21, 22) (Fig. 12A). The four mutants were selected as those that exhibited the greatest differences in epiphytic population sizes relative to the wild type in laboratory assays. The Trp auxotroph consistently exhibited the poorest ability to survive and grow on bean leaves under all conditions examined. In one field experiment in which population sizes of the mutants were monitored for 8 days following inoculation, the behavior of the mutants differed relative to each other and to the wild type, with the differences being greatest about 4 days after inoculation (Fig. 12A).

The Met auxotroph has been characterized at the genetic level. Cloning and sequencing of the affected DNA locus yielded two consecutive open reading frames (ORFs). One (*metX*) encodes a protein with homology to several homoserine *O*-acetyltransferases that function in methionine biosynthesis in several different organisms. No similarity was found between the sequence of the second ORF (*metW*) to sequences in the databases. Although Tn5 had inserted into *metX*, the first of the two ORFs, it appears that both genes are required for wild-type epiphytic fitness (7). Exogenous methionine was applied to leaf surfaces to determine whether the reduced fitness of the Met auxotroph could be due to limiting methionine concentrations. Although small increases in population sizes of the mutant and B728a were found on leaves maintained under moist conditions, application of methionine did not increase the survival of the strains under dry (stressful) conditions. Hence, methionine prototrophy appears to be required for optimum fitness of B728a, but the underlying mechanism is not known at this time.

Perhaps as informative as what was found is what was not found in the search for mutants affected in their abilities to survive and grow on leaf surfaces (186). All of the mutants were similar to the parent strain in their ability to utilize the 31 different organic compounds tested. Pigment production has long been hypothesized as a trait that enables epiphytic bacteria to tolerate UV irradiation on leaf surfaces. However, all mutants produced a fluorescent pyoverdine siderophore, sim-

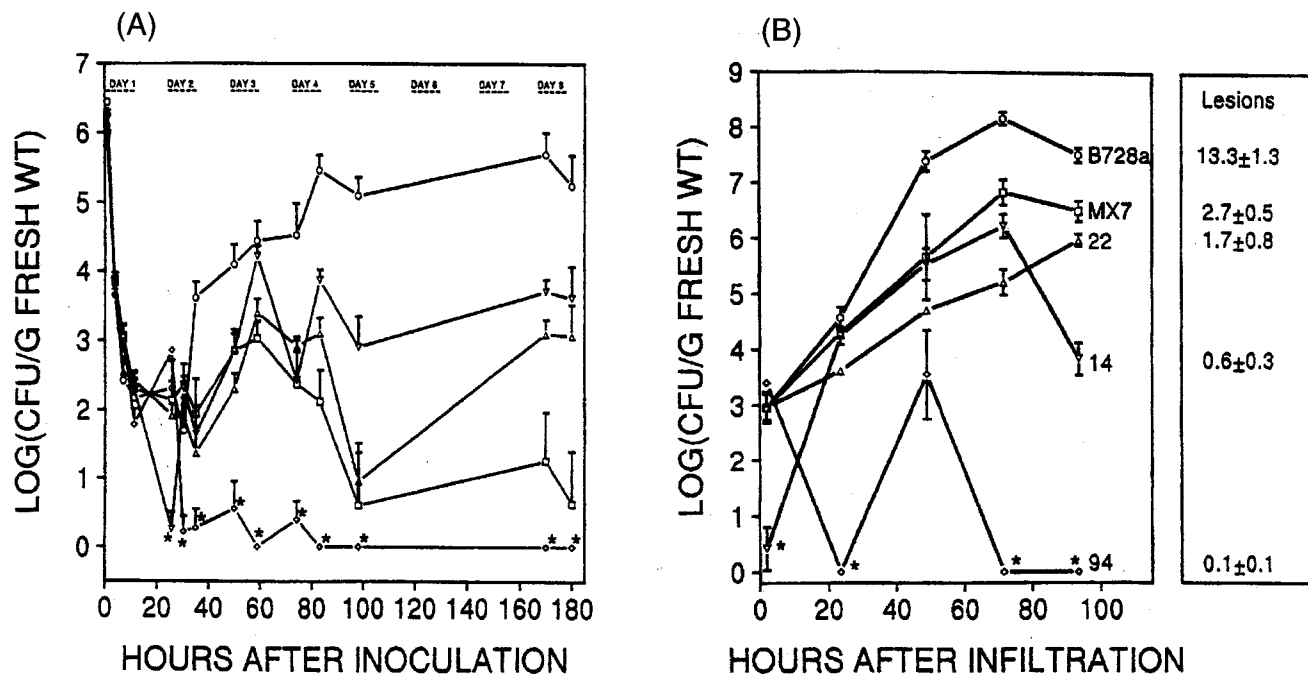


FIG. 12. (A) Population dynamics of *P. syringae* pv. *syringae* B728a (○) and Tn5 mutant derivatives 14 (▽) and 22 (△), methionine auxotroph MX7 (□), and tryptophan auxotroph 94 (◇) on bean plants under field conditions in June. Each point represents the mean and SE of 12 leaf samples. Periods of daylight are indicated by dashed lines. Because means could not be estimated for data sets with 10 or more leaves harboring undetectable populations, means for these data sets (*) were calculated using log (CFU per gram [fresh weight]) values of 0 for leaves with no detectable population. Reprinted from reference 22 with permission. (B) Population dynamics of *P. syringae* pv. *syringae* B728a (○) and Tn5 mutant derivatives 14 (▽) and 22 (△), methionine auxotroph MX7 (□), and tryptophan auxotroph 94 (◇) after vacuum infiltration into the intercellular spaces of leaves. Each point represents the mean and SE of eight samples, each composed of two 6.5-mm leaf disks. The values indicating the number of lesions induced by each strain are the mean and SE of seven plants, with the lesion number per plant represented by the mean number of lesions enumerated in three randomly chosen 1.2-cm² leaf regions. Reprinted from reference 21 with permission.

ilar to the wild type. Indeed, no evidence could be found for *in situ* production of fluorescent pigments by *P. syringae* on bean leaf surfaces, as assessed in growth chamber experiments (199). The behaviors of a few mutants would appear to support the role of previously hypothesized fitness traits. Four of the mutants were reduced in motility, while two were nonmotile, as assessed on a semisolid medium. Findings from previous research with chemically induced nonmotile mutants suggested that motility contributed to the epiphytic fitness of *P. syringae* as assessed in laboratory experiments (100). Some of the mutants exhibited reduced osmotolerance, as measured by their ability to grow in medium containing high NaCl concentrations. A few mutants appeared to be reduced in production of extracellular polysaccharide (EPS), as assessed by colony morphology, but none were apparently devoid of EPS production. Alginate, an EPS produced by *P. syringae*, has been implicated as an epiphytic and virulence factor based on measured reductions in population sizes and disease severity of an alginate-deficient mutant as assessed under laboratory conditions (323).

Epiphytic fitness is undoubtedly influenced and affected by a number of traits and genes. With mutants that exhibit pleiotropic phenotypes, it may be difficult to ascribe causality to a phenotype. Cloning and sequencing of the affected loci of the large pool of epiphytic fitness mutants obtained from the Tn5 mutant hunt may reveal the nature of the genes affected that appear to contribute to epiphytic fitness.

A final point to be made is that phenotypic characterization of the four mutants that exhibited the greatest reduction in epiphytic fitness relative to the wild type revealed that all the mutants were indeed reduced in their ability to survive the

conditions deemed stressful. However, in laboratory assays, the mutants were also reduced to different degrees in net growth on leaves kept moist (i.e., presumably nonstressful) and in their ability to grow when infiltrated into leaves (Fig. 12B). Hence, the mutants would appear to be generally affected in their ability to grow and survive in and on leaves. One is thus left with the conclusion that they are not strictly "epiphytic" mutants. This conclusion does not diminish the significance of the findings but rather provides additional information as to the possible effects that specific genes and traits may play in the interactions of *P. syringae* on the surface and in the apoplast of leaves. From the perspective of *P. syringae*, the leaf surface may not be as distinct a place to live compared to the apoplast as we may think. That some (many) of the epiphytic fitness genes may turn out to be what we tend to think of as "ordinary" housekeeping genes should not be surprising. Surely both the function and regulation of such housekeeping genes should be tuned to operate optimally in the organism's favored habitat—leaves.

The ability to attach or adhere to surfaces is a trait that is often mentioned in regard to bacterial fitness in a number of habitats. Although attachment per se is difficult to define in the context of the highly variable leaf habitat, mutants of *P. syringae* lacking pili have been shown to be more readily washed from leaves in short-term laboratory experiments (239–242, 274). In field experiments, mutants of *P. syringae* pv. *tomato* lacking type IV pili achieved slightly lower population sizes on leaves of tomato plants than those of the parental wild-type strain DC3000 (236). Thus, the presence of pili appears to affect the fitness of *P. syringae* in the phyllosphere.

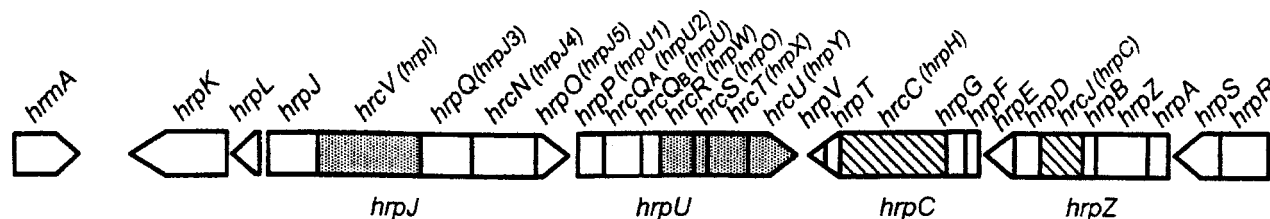


FIG. 13. Organization of the *hrp* gene cluster in *P. syringae* pv. *syringae* strain 61. The gene designation employs the unified nomenclature for widely conserved *hrp* genes (*hrc*) (27). Arrowheads indicate the direction of transcription for each operon. Genes encoding proteins predicted to be associated with the inner or outer membrane of the type III secretion system are stippled and hatched, respectively, but HrcJ may be associated with both membranes. The cluster of *hrp* genes from *hrpK* to *hrpR* is similar in strains 61 (weakly virulent) and B728a (highly virulent). Flanking regions are not as highly conserved in the two strains. Reprinted from reference 35 with permission.

Genes Associated with Pathogenicity

The substantial efforts to elucidate the molecular mechanisms of pathogenicity and virulence in *P. syringae* and other phytopathogenic bacteria have led to the identification of a large number of genes that in some way affect the ability of the bacteria to cause disease (reviewed in reference 4). Much of what is known about the phenotypes attributed to these genes is based on laboratory assays in which the bacteria are infiltrated into leaves of plants. Such methods circumvent a significant portion of the natural interactions that occur between bacteria and plants in the field. Yet even in these assays, symptom development (or lesion formation) is often not the only phenotype affected in many of the mutants that have been identified. For example, in many cases mutants that no longer cause a pathogenic reaction are also impaired in their ability to grow to wild-type levels in such assays.

The pleiotropic phenotypes of nonpathogenic mutants identified thus far precludes an assessment of the contribution of disease causation per se to overall fitness of *P. syringae*. However, the contribution of specific genes (not phenotypes) to fitness can be examined. Furthermore, we may be able to ask to what extent impaired plant-associated growth might contribute to a mutant's impaired ability to cause disease. From the dose-response experiments of Lindemann et al. (167) and Rouse et al. (246), the probability of brown spot disease occurring on snap bean plants in the field is a function of population sizes of *P. syringae* pv. *syringae*. Hence, any gene(s) that affects pathogen population sizes will also necessarily affect disease. For a system such as the *P. syringae*-leaf ecosystem, there is a nearly limitless supply of test subjects (i.e., plants and leaves) that can be manipulated in a natural setting—the field. Hence, the system has a distinct experimental advantage over, say, animal or human pathogens, in attempts to bridge the gap between what is known about the molecular biology of pathogenicity-associated genes and the ecology and epidemiology of the pathogen of interest.

In this section, we focus on two groups of pathogenicity-associated genes for which there is some information on the field behavior of mutants bearing defects in genes representative of the groups. We discuss how information from experiments conducted in the field can be used to better understand the functions of these genes in plant-bacterium interactions while simultaneously gaining insight into their impact on the field fitness of a bacterium for which disease causation is but one aspect of its life strategy.

Genes in the *hrp* regulon. *hrp* (hypersensitive reaction and pathogenicity) genes were originally isolated from *P. syringae* pv. *phaseolicola*, the causal agent of halo blight on bean (173). The salient phenotypes of *hrp* mutants include an inability to elicit the HR on nonhost plants and resistant cultivars of sus-

ceptible hosts, an inability to cause disease on host plants, and an inability to grow to wild-type levels when infiltrated into leaves in laboratory assays. The HR, which is manifested by rapid collapse and death of plant cells, is thought to arrest pathogen growth and spread, leading to an incompatible reaction (87). Although the HR is believed to be an important component of disease resistance, whether this is indeed the case remains unclear at this time (322). Note that inability to cause the HR on a nonhost such as tobacco does not render an *hrp* mutant of *P. syringae* pathogenic to the nonhost.

Since the initial report by Lindgren et al. (173), *hrp* genes have been isolated and characterized from strains representative of nearly all major genera of gram-negative plant-pathogenic bacteria (reviewed in references 4, 5, 28, 88, 101, 102, 132, 133, 172, and 305). Genes with similarity to *hrp* genes have recently been found in a strain of *P. fluorescens* (230). This is highly significant because this bacterium is not a plant pathogen. Indeed, the specific strain of *P. fluorescens* examined is a colonist of the rhizosphere, with apparent plant growth-promoting activity.

hrp genes in plant-pathogenic bacteria are present as clusters spanning roughly 25 kb (Fig. 13). Putative functions of the proteins encoded by *hrp* genes include regulation of transcription, components of a secretion pathway, and proteins that are secreted via this pathway (reviewed in references 4, 5, 89, 102, 132, 133, and 172). The finding that a subset of *hrp* genes has similarity to genes that encode components of the novel type III secretion system present in animal pathogens such as *Yersinia*, *Shigella*, and *Salmonella* spp. has generated much interest in this group of genes (5, 15, 83, 102, 211). In animal systems, virulence effector molecules are exported via the type III secretory pathway, and in some cases, these molecules may pass directly into the cytoplasm of the target host cells in response to pathogen-host cell contact (reviewed in references 40, 41, 82, 83, 131, 162, and 204). Nine *hrp* genes that bear sequence similarity to genes that encode components of this pathway have been redesignated *hrc* genes (for HR conserved) to reflect the conserved nature of these genes among plant and animal pathogens (27). Under growth conditions that induce *hrp* gene expression, *P. syringae* pv. tomato strain DC3000 produces pili (237, 238). That the major structural proteins of these pili is coded by *hrpA* has led to the speculation that Hrp pili may serve as the conduit for direct delivery of effector proteins through the plant cell wall and into the cytoplasm.

Most of the molecular research on *hrp* genes has focused on their function in elicitation of the HR. Little is known about the ways in which the Hrp secretion system and its associated effector molecules contribute to pathogenicity. Although it has long been clear that *hrp* mutants do not grow well in leaves of susceptible hosts (173), the possibility that this inability to grow

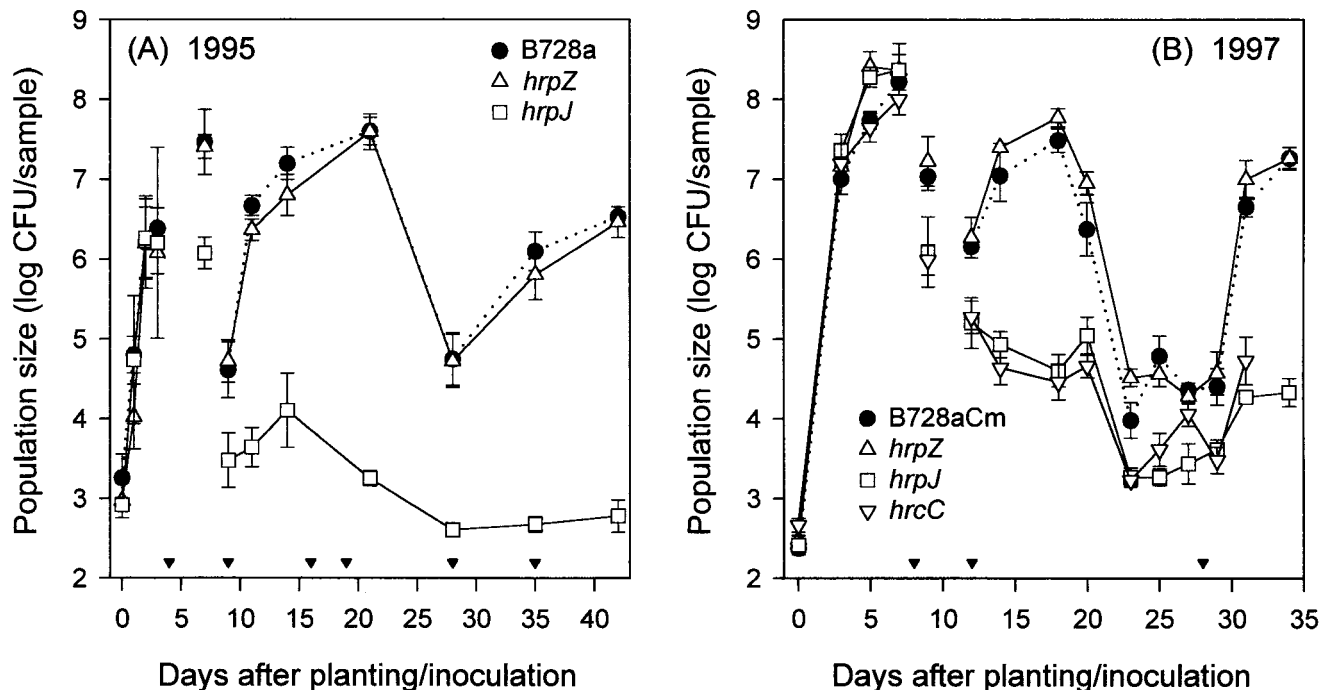


FIG. 14. Population dynamics of *P. syringae* pv. *syringae* B728a *hrp* mutants ($\Delta hrpZ::nptII$, $hrpJ::\Omega Spc$, and $\Delta hrcC::nptII$) in association with field-grown snap bean plants. The bacterial strains were inoculated onto seeds immediately before planting. Samples were seeds or germinating seedlings collected on or before days 7 (A) and 9 (B) after planting; primary leaves were collected between 9 and 14 (A) and 12 and 20 (B) days after planting; and single leaflets from trifoliolate leaves were collected at all other times. Each datum point represents the mean log CFU per sample and SE based on three (A) or four (B) replicate plots with six or eight individual samples per plot. Inverted solid triangles indicate rain events with sustained rates of $>1 \text{ mm min}^{-1}$. Reprinted from reference 106 with the permission of the publisher.

might explain the requirement for *hrp* genes in pathogenicity has been largely ignored.

What can be learned about the role(s) of *hrp* genes and the type III secretion system in pathogen-host interactions by examining these interactions across many bacterial generations as they occur in the "real world" with its fluctuating, variable, and dynamic environment? To address this question, we field-tested three *hrp* mutants of *P. syringae* pv. *syringae* strain B728a ($\Delta hrpZ::nptII$, $\Delta hrcC::nptII$, and $hrpJ::\Omega Spc$) (106) (Fig. 14). *hrpZ* encodes a protein that is secreted via the Hrp type III secretion system (103). HrcC is thought to function as an outer membrane component of the Hrp secretion system (35, 53, 129, 130), similar to YscC in *Yersinia* spp. (154). The *hrpJ* mutant carried a polar mutation in *hrpJ*, the first of five genes in the *hrpJ* operon. One or more of the genes in the operon appears to function as an inner membrane component(s) of the secretion system (35).

When inoculated onto bean seeds at the time of planting in the field, the *hrp* mutants behaved similarly to the wild-type B728a before plant emergence (Fig. 14) (106). All strains grew rapidly on germinating seeds and developing preemergent seedlings. However, phyllosphere population sizes of the *hrp* secretion mutants but not the *hrpZ* mutant were significantly reduced relative to that of B728a. The absence of substantial population increases during rainy periods favorable for growth of the wild-type strain suggested that the Hrp type III secretion system plays a critical role in enabling *P. syringae* to grow in the phyllosphere. Indeed, some *hrp* genes they may be considered fundamental phyllosphere growth (and possibly survival)-enabling genes that have a major impact on the field fitness of *P. syringae*. If the behavior of the secretion mutants is attributable primarily to defects in the Hrp type III secretion apparatus, then the requirement of the system for growth of *P. syringae*

appears to be habitat and/or environment specific. The system is necessary for *P. syringae* to flourish in the phyllosphere but not in association with below-ground germinating seeds and developing preemergent seedlings.

The *hrp* secretion mutants caused brown spot lesions on primary bean leaves in the field (106). In the experiment shown in Fig. 14B, population sizes of the mutants and wild type were unusually large on below-ground plant parts. At the first sampling of primary leaves 12 days after planting, a few leaves harbored population sizes that were sufficiently large ($>10^6$ to 10^7 CFU per leaf) to be predictive of disease for strains capable of causing brown spot lesions. At this time, all leaves sampled were visibly asymptomatic. Two days later, brown spot lesions were detected in the plots (disease incidence of about 4% for the secretion mutants and 63% for the wild type). Characterization of bacteria isolated from the lesions confirmed that the *hrcC* and *hrpJ* mutants were the causal agents of the lesions detected in the field plots inoculated with these strains. The small amounts of disease caused by the secretion mutants are generally consistent with the dose-response experiments of Rouse et al. (246). The observation that *hrp* mutants are able to cause some disease in the field is also consistent with findings reported in the initial publication on *hrp* genes (174). Growth of various *hrp* mutants of *P. syringae* pv. *phaseolicola* as assessed in leaf infiltration assays was quantitatively different (174). The mutants grew to different extents relative to the wild type and in some cases caused correspondingly different amounts of disease (halo blight of bean), in some respects analogous to the behavior of the epiphytic fitness mutants (see Fig. 12B). Although this piece of the puzzle has been largely cast aside for over a decade, it is time that it be picked up once again and given careful reconsideration.

The role of the type III secretion systems in growth of a

bacterial pathogen is not without precedent. Shea et al. (265) reported that the type III secretion system within *Salmonella enterica* serovar Typhimurium pathogenicity island 2 (SPI2) plays a role in growth of this pathogen. A mutation in *ssaI*, a gene encoding a component of the SPI2 type III secretion system, affected growth but not survival of the pathogen in a mouse model system. How might the type III secretion system and its effector molecules affect growth of *P. syringae* in the phyllosphere? The answer is not yet within our grasp. However, a few pieces of information are worthy of mention. Atkinson and Baker (10, 11) previously reported that multiplication of *P. syringae* pv. *syringae* in the apoplast following infiltration of the bacterium into bean leaves was associated with the ability of the bacterium to induce a host plasma membrane K^+/H^+ exchange. According to their model, growth of the pathogen is due to leakage of sucrose from plant cells, which in turn is brought about by an increase in the pH of apoplast fluid (from about 5.5 to 7.5). Alkalinization of the apoplast, as measured by monitoring the pH of solutions containing bean leaf disks inoculated with the pathogen, resulted in turn from the K^+/H^+ exchange. The impaired in planta growth ability of *hrp* mutants correlated with their inability to induce the ion exchange. Secretion of some proteins via the type III system in *P. syringae* (284), *X. campestris* (245), and serovar Typhimurium (25) has recently been reported to be pH dependent (i.e., stimulated at pH 5 to 6, depending on the system). Only time will tell whether these pieces of information will eventually fit into the puzzle of how the type III secretion system affects the growth of *P. syringae* in the phyllosphere.

Current mechanistic models for the function of *hrp* genes suggest that pathogenicity or virulence factors may traverse the Hrp type III pathway following intimate interactions of the pathogen with plant cells within the apoplast of leaves (4, 37, 211). However, to our knowledge, there is no case for which the effects of *hrp* genes on disease development have been demonstrated to be distinct from effects on growth in or on leaves. The possibility that the role of the type III secretion system in pathogenicity is mediated by its requirement for growth of *P. syringae* in the phyllosphere is a plausible alternative to current models. Furthermore, it appears likely that the Hrp secretion system is required for growth of *P. syringae* on leaf surfaces, not only in the apoplast. In its present form, the model contains elements that are difficult to reconcile with those early interactions between plant and bacterium which most likely occur at the plant surface rather than in the apoplast. Regardless of the underlying mechanism by which the Hrp type III secretion system affects interactions of *P. syringae* with leaves, the *hrp* regulon appears to play a fundamental and important role in the overall life strategy of the bacterium. Indeed, recent phylogenetic studies demonstrate that *hrpL* and *hrpS* (*hrp* genes with regulatory functions) appear to be as evolutionarily stable among pathovars of *P. syringae* as are *gyrB* and *rpoD*, two basic housekeeping genes (256).

If genes in the *hrp* regulon are not directly required for symptom development by *P. syringae*, what genes enable the bacterium to cause lesions?

Genes in the *gacS* regulon. Willis et al. have identified a number of genes that are required for brown spot lesion formation in *P. syringae* pv. *syringae* strain B728a (127, 151, 152, 233, 234, 304, 306; D. K. Willis, J. J. Holmstadt, A. K. Savage, C. A. Hinckley, J. L. McEvoy, and T. G. Kinscherf, abstr., *Phytopathology* **89**:S85, 1999). Unlike *hrp* genes, these genes are not required for elicitation of the HR. The first of these to be identified and extensively characterized is *gacS* (global activator sensor kinase). The gene was initially named *lemA* because of its requirement for lesion formation. However, with

the finding that many other phenotypes are affected in *gacS* mutants, the gene was renamed to better reflect its requirement in *P. syringae* (151). GacS is similar to the transmembrane histidine kinase portion of a family of bacterial two-component regulatory systems (127). In these systems, the kinase is thought to serve as a sensor of environmental stimuli. The signal is then relayed by phosphorylation of the cytoplasmic response regulator, which in turn mediates changes in transcription (reviewed in references 124 and 221). The *gacA* gene has been identified genetically as encoding the cognate response regulator in *P. syringae* pv. *syringae* B728a (233, 306). The *gacA* gene was first described for *P. fluorescens* strain CHA0 as a global regulator of production of a number of secondary metabolites that contribute to the bacterium's biocontrol activity against a fungal root rot disease of tobacco (160).

Unlike *hrp* genes, the requirement for *gacS* in *P. syringae* appears to be pathovar specific (232). In *P. syringae* pv. *syringae*, the gene is required for lesion formation (brown spot disease of bean) and toxin production (syringomycin and syringopeptin). In *P. syringae* pv. *coronafaciens*, *gacS* is required for toxin production (tabtoxin) but not lesion formation (halo blight of oats) (17). In *P. syringae* pv. *tomato* (bacterial speck of tomato; the toxin is coronatine) and *P. syringae* pv. *phaseolicola* (halo blight of bean; the toxin is phaseolotoxin), mutations in *gacS* affect neither lesion formation nor toxin production (232). Homologs of the *gacS* and *gacA* genes have since been found in a number of other pseudomonads (e.g., *P. fluorescens*, *P. viridiflava*, and *P. aeruginosa*) (17, 34, 39, 81, 97, 160, 165, 231, 254, 301; S. T. Lam, T. D. Gaffney, R. A. Frazelle, K. Gates, J. Di Maio, N. Torkewitz, J. Ligon, S. Hill, S. Goodwin, and H. J. Kempf, abstr., *Mol. Ecol.* **3**:620, 1994) and in *Erwinia carotovora* (73, 79), where they appear to affect bacterium-plant or bacterium-microbe interactions. In *Salmonella* serovar Typhimurium, the *gacA* homolog *sirA* has been shown to affect regulation of type III secretion systems present in this animal pathogen (3, 137). Similarly, the GacS and GacA counterparts BvgA and BvgS that control expression of virulence factors in *Bordetella bronchiseptica* appear to additionally regulate a type III secretion system in this animal pathogen (324). Thus, at least in these animal pathogens, a link between the equivalent *hrc* and *gacA* homologs has been made. Such a connection between the *gacS/gacA* and *hrp* regulons has been assumed not to occur in *P. syringae* because of the HR^+ phenotype of *gacA* mutants. It will be interesting to learn if such a connection exists in *P. syringae*.

In addition to brown spot lesion formation and production of the toxins syringomycin and syringopeptin, *gacS* and *gacA* mutants of strain B728a are affected in production of extracellular proteases, acyl homoserine lactone, and alginate and in swarming behavior on soft agar medium (62, 128, 150, 151, 233; Willis et al., abstr., 1999) (Fig. 15). Unlike *hrp* mutants, *gacS* and *gacA* mutants are able to cause the HR in tobacco, and most significantly, they are fully able to grow as well as the wild type when infiltrated into leaves of bean plants (Fig. 16A). That *gacS* and *gacA* do not affect the in planta growth of *P. syringae* pv. *syringae* is unique among the many mutants of this pathogen that have been described as being nonpathogenic or reduced in virulence. Furthermore, that *gacS* and *gacA* mutants are indistinguishable from the wild type in their ability to multiply when infiltrated into leaves but are unable to cause disease demonstrated that bacterial growth in the apoplast is not sufficient for brown spot lesion formation (233, 304). The significance of this finding cannot be overemphasized.

A number of genes that are regulated by GacS and GacA have been identified (151; Willis et al., abstr., 1999). Among

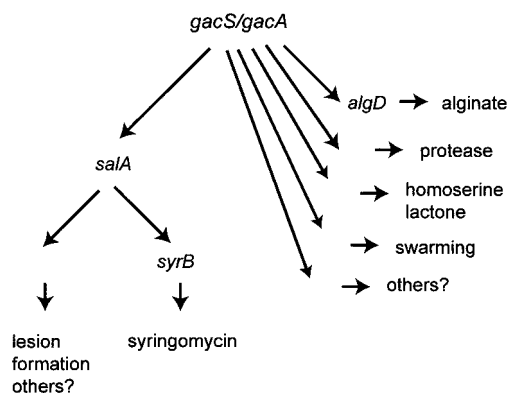


FIG. 15. *gacS/gacA* regulon in *P. syringae* pv. *syringae* B728a and phenotypes affected.

these is *salA* (syringomycin and lesion formation) (151). Sequence analysis of *salA* suggests that it encodes a DNA-binding protein with little similarity to other proteins in the current databases. In laboratory assays, a *salA* mutant is impaired only in lesion formation and production of syringomycin. A *salA* mutant is similar to the wild type in production of extracellular proteases, acyl homogoserine lactone, and alginate (151). Kitten et al. (151) have demonstrated that SalA positively regulates expression reporters inserted into the syringomycin biosynthesis gene *syrB*. A model for the *gacS/gacA* regulon places *gacS/gacA* at the top of a regulatory hierarchy in B728a. *salA* is regulated by GacS and GacA and in turn plays a regulatory role in activating the expression of a subset of genes in the regulon, including genes necessary for lesion formation and syringomycin production (Fig. 15).

In field experiments, population sizes of a *gacS* mutant were

significantly reduced relative to B728a in association with bean leaves (Fig. 16B), but not with germinating seeds, similar to the *hrp* secretion mutants (110). Because a *gacS* mutant is able to grow when infiltrated into bean leaves in laboratory assays, it is tempting to speculate that a subset of genes regulated by *gacS* may be affecting the epiphytic fitness of B728a under field conditions. In this regard, it is interesting to note that *gacS* plays a role in regulating production of the exopolysaccharide alginate in *P. syringae* pv. *syringae* (Willis et al., abstr., 1999) and *P. viridiflava* (165). Alginate has been reported to contribute to the virulence and epiphytic fitness of *P. syringae* pv. *syringae* strain 3525, as measured in growth chamber experiments (323). The fitness of alginate-deficient mutants under field conditions remains to be examined.

gacS, *salA*, and *syrB* function at different tiers in the *gacS* regulon. GacS regulates expression of *salA*. SalA, in turn, regulates expression of the syringomycin biosynthesis gene *syrB*. In a field experiment in which the fitness of the mutants was compared to that of wild-type B728a and to each other, phyllosphere population sizes of the *gacS* mutant (*gacS::Tn5*) averaged roughly 125-fold less than wild-type population sizes (range, 7- to 1,250-fold over time from plant emergence to pod set), while population sizes of the *salA* mutant averaged only about 5- to 6-fold less than wild-type population sizes (range, 2- to 20-fold) (S. S. Hirano, D. K. Willis, and C. D. Upper, abstr., Phytopathology 89:S34, 1999). Hence, the overall difference in population sizes was greater between the *salA* and *gacS* mutants than between the *salA* mutant and the wild type. The data suggest that the subset of genes in the *gacS* regulon that are not regulated by *salA* play an important role in growth and survival of *P. syringae* pv. *syringae* in association with leaves in the field, in some respects similar to *hrp* genes. The *salA* mutant established population sizes that were sufficiently large to be predictive of disease but did not cause the expected amount of disease in the field (Hirano et al., abstr., 1999).

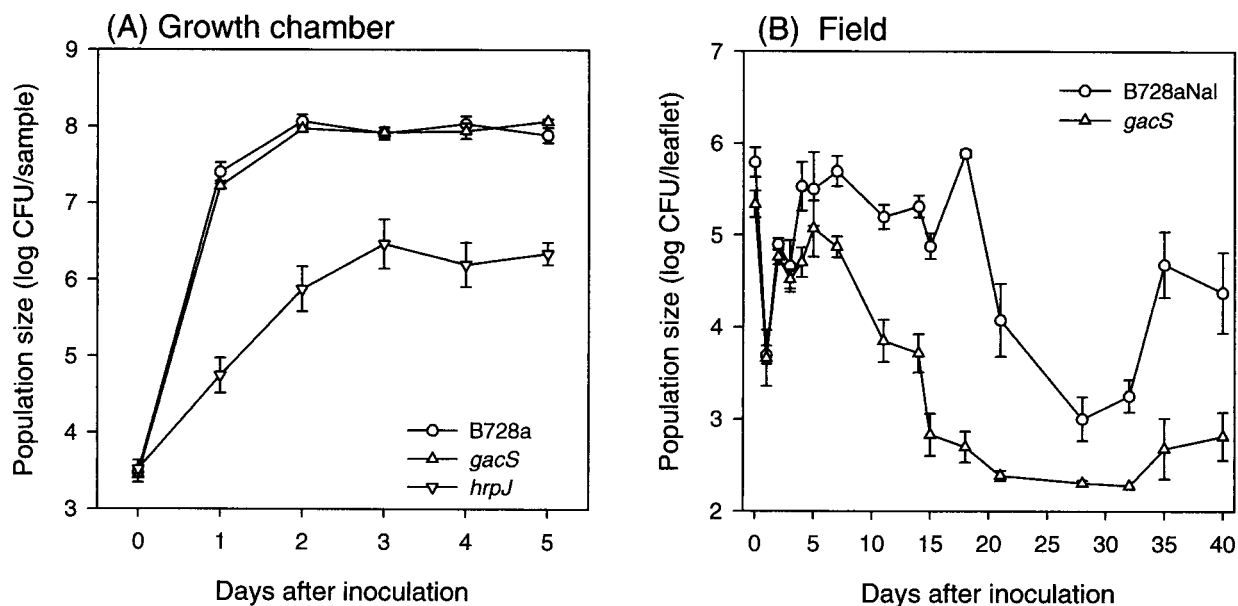


FIG. 16. Population dynamics of a *gacS* mutant of *P. syringae* pv. *syringae* B728a. (A) Bacterial strains (10^6 CFU/ml) were infiltrated into primary leaves of growth chamber-grown bean plants. At each sampling time, leaf disks (6-mm diameter) were removed with a cork borer from each of five leaves. Each datum point represents the mean log CFU per sample and SE ($n = 5$). Unlike the *hrpJ* mutant, growth of the *gacS* mutant was indistinguishable from that of the wild type. (B) Population dynamics of a *gacS* mutant under field conditions. The bacterial strains were inoculated onto the foliage of 25-day-old snap bean plants. Population sizes were estimated by dilution plating of leaf homogenates. Each datum point represents the mean log CFU per sample and SE based on three replicate plots with eight individual leaf samples per plot. Reprinted from reference 110 with the permission of the publisher.

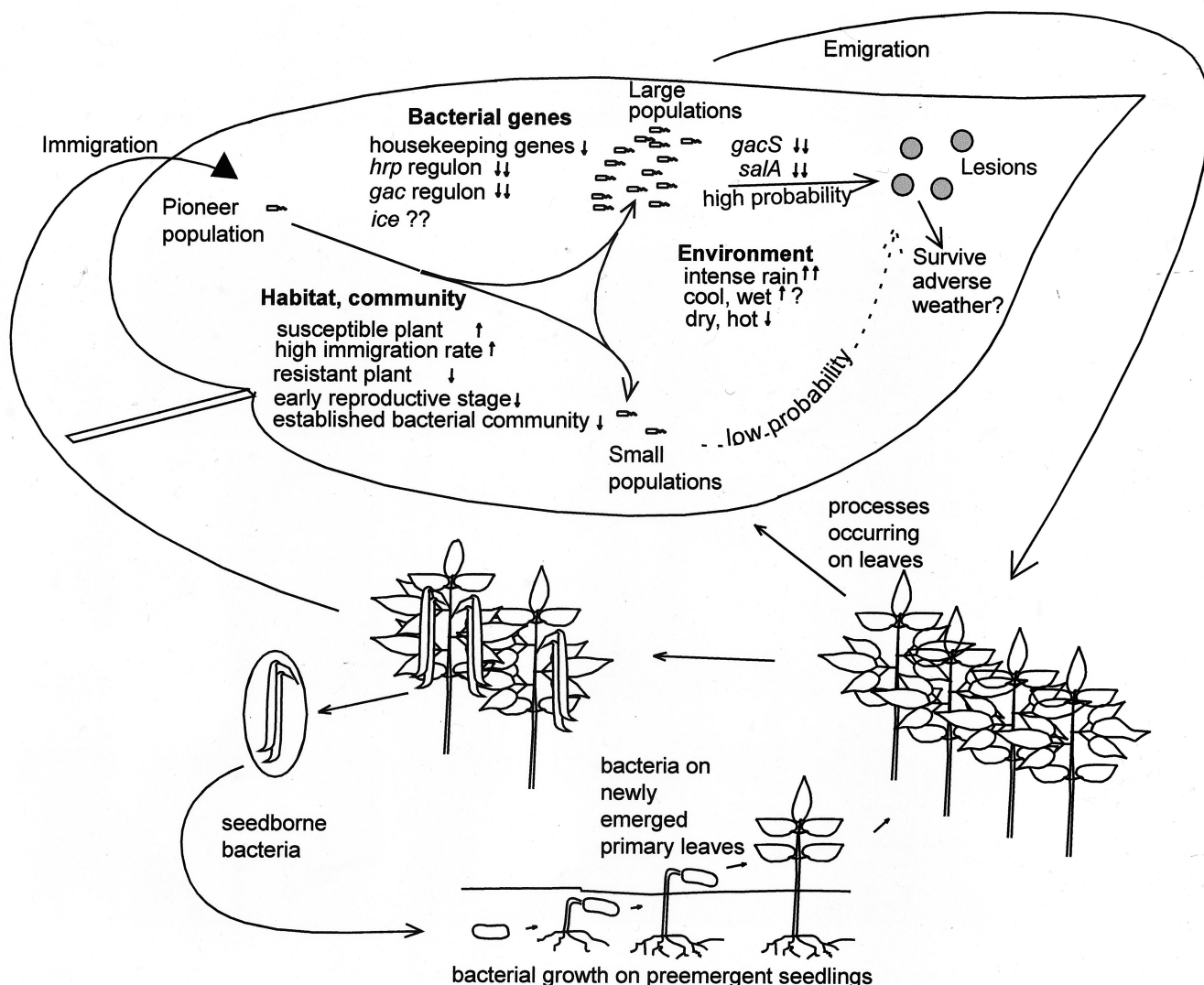


FIG. 17. Interactions of *P. syringae* and leaf habitats—putting the puzzle together. An immigrant arrives on a leaf, either from growth on a germinating seed (bottom) or through the air. Conducive conditions such as a susceptible plant, intense rain, and early vegetative stage of plant development favor growth and establishment of large population sizes of *P. syringae*. Dry, hot conditions or an unfavorable plant will diminish growth and drive bacteria toward survival rather than growth. Some bacterial genes in the *gac* and *hrp* regulons and several housekeeping genes are necessary for sufficient growth to achieve large bacterial population sizes. Genes necessary to produce type IV pili may also enhance bacterial growth and survival. The larger the population size, the more bacteria are available to emigrate, either on insects when leaves are wet or as aerosols in dry sunny weather, or to be washed off by rain. As population sizes approach 10^6 cells per leaflet, disease becomes likely. Genes in the *gac* regulon (*gacS/gacA* and *salA*) are required for lesion formation. Lesions may provide a site for enhanced survival of *P. syringae* during unfavorable weather. These processes are repeated throughout the growing season on each of the 10^6 or more leaf habitats per hectare, with bacterial generation times on the order of 2 to 5 h. If the plants are allowed to mature, bacteria present on pods may move to seeds, where the bacteria can survive until the seeds are planted to begin the next plant generation. ↑ and ↓, positive and negative effects, respectively.

Hence, the contribution of lesion formation to population sizes can be no more than the difference between the *salA* mutant and the wild type (average of 5- to 6-fold and range of 2- to 20-fold). Population sizes of the *syrB* mutant were roughly two- to threefold less than that of the wild type. The *syrB* mutant caused brown spot disease in the field; disease incidence was slightly reduced relative to the wild type. Hence, approximately half of the reduction in populations of the *salA* mutant relative to the wild type may be due to its inability to produce syringomycin. However, loss of syringomycin production did not explain the impaired ability of a *salA* mutant to cause brown spot lesions. Pursuit of the genes that are regulated by *salA* and may play a direct role in brown spot lesion is ongoing in the laboratory of D. K. Willis and colleagues.

The experiments described above have shown us that genes in the *hrp* and *gacS* regulons (and likely many others) play major roles in interactions of *P. syringae* with plants in the field. Characterization of these genes at the molecular level has progressed substantially in recent years, largely as two distinct regulons, each said to be required for pathogenicity in *P. syringae*. Both regulons affect the ability of the pathogen to grow in and on leaves in the field, an activity essential for disease epidemics to occur. Genes in the *gacS* regulon, in particular genes regulated by SalA, a key branch point in the regulon, appear to be directly required for lesion formation in the *P. syringae* pv. *syringae*-snap bean-brown spot system. It is useful to consider the function of genes in both regulons (and others

as they are identified) in the integrative context of the interactions of *P. syringae* with plants in the field.

The field setting has provided useful information and new insight on the role(s) that pathogenicity-associated genes play in interactions of *P. syringae* and its susceptible host, bean plants. However, there remains an important caveat. Until a plant-associated phenotype (e.g., decreased population sizes) exhibited by a mutant can be restored to wild-type levels, we must be cautious in attributing the altered phenotype to the gene specifically mutated. The duration of our field experiments renders restoration in *trans* (i.e., wild-type copy on plasmid in mutant background) problematic due to possible plasmid instability over the many hundreds of bacterial generations that occur during these experiments. We have attempted to address this problem in several ways. For example, the experiments with the *hrp* secretion mutants involved two constructs in which the type III system was similarly affected, although different genes required for a functional system were mutated. We can more rigorously test the effects of mutations in *gacS* and other genes in the regulon by examining the behavior of constructs in which a specific gene has been mutated in different ways, thus demonstrating the reproducibility of observed phenotypes.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

We have provided a brief tour of many, but surely not all, of the multitude of facets of the ecology of bacteria in the phyllosphere. Throughout we have used *P. syringae* as a model to provide examples of what is known of the various processes that influence the behaviors of bacteria associated with leaves. It is not yet clear whether the behavior of *P. syringae* in association with snap bean leaves is a model for plant-bacterium interactions or just a case study (120). This issue will only be resolved when the ecology of other bacterial species in the phyllosphere is understood as well as is the *P. syringae*-bean leaf system.

The diagram in Fig. 17 illustrates a number of the factors affecting the ecology of *P. syringae* on snap bean leaves, as discussed earlier in this review. These are pieces that are fairly well understood and that would seem to us to belong where they have been placed in this puzzle. Two points are not adequately made by Fig. 17, however. First, many pieces of the puzzle have been omitted because of our ignorance. Even if the *P. syringae*-bean leaf system is the best understood of phyllosphere-bacterium interactions at this time, there is undoubtedly far more to learn than we already know of how this puzzle fits together. Second, the model displays only two dimensions, whereas interactions in the field are more accurately represented in multidimensional space. By the time that plants are fully grown, there will be as many as 7×10^7 leaf habitats on each hectare planted to commercial snap beans. Each of the physical and biotic factors affecting bacterial population dynamics will vary across the field and within the bean canopy. Thus, the stochastic nature of the development of populations of bacteria on populations of leaves is not represented in the figure. Furthermore, regional effects on these processes are not represented. Bacteria emigrating from and immigrating to a particular field reflect what is happening in other fields. Those nearby will have a greater effect than more distant fields, yet an overall regional influence exists.

As illustrated in Fig. 17, lesion formation is only one of the many activities of *P. syringae* in association with bean leaves. Based on comparison of behaviors of various mutants in the field, the competitive advantage that lesion formation affords this bacterium is not as great as expected and may turn out to

be quite small. The effects of bacterial genes required for growth in the phyllosphere and of physical and other biotic factors on the relative success of this bacterium are far greater than the effect of not being able to form a lesion. Thus, if we wish to understand the mechanisms associated with successful disease causation in the field, it is far more important to understand the processes that lead to development of large population sizes of this bacterium than to understand the mechanism by which lesions are formed per se.

We hope that we have left the reader with a clear idea of the importance of bacteria in the phyllosphere and the need to learn more about these fascinating, complex, highly interactive systems. There is so much yet to learn that there is ample room for greatly expanded efforts on these interesting problems from disciplines as diverse as genetics, ecology, and meteorology.

The powerful tools of molecular biology have greatly advanced our understanding of plant-microbe interactions at the molecular level. Such studies, of course, are no stronger than the limitations provided by the biological assays upon which they depend. Regardless of the sophistication of the molecular mechanisms proposed, they are of little value until a relationship is tightly established between the mechanisms and the phenomena that they are thought to explain. At the moment many proposed mechanisms are available for bacterium-plant interactions in the phyllosphere, but few, if any, have been clearly shown to explain (or even be related to) the phenomena which they are supposed to explicate. For examples, the role of pigmentation in fitness of phyllosphere bacteria, the roles of specific genes thought to affect plant-bacterium interactions, the role of nutrients or other mechanisms for regulating bacterial population sizes associated with leaves, the importance of immigration in a growth-driven system, and many other issues as discussed above clearly deserve careful examination. We believe it is time to devote greater effort to the biological side of these systems, to do the definitive experiments that will "close the loop" and create the necessary links between mechanisms and phenomena. Unfortunately, at least some of this work will necessarily have to be done in that most difficult of all laboratories—the field.

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