Construction and use of bacterial sensors to investigate the role of water deprivation in bacterial-plant interactions

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Construction and use of bacterial sensors to investigate the role of water deprivation in bacterial-plant interactions

by

Catherine Axtell Wright

A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

Major: Microbiology

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For the Major Program
for my husband
for my mother and father
for my brothers
and for myself
General Introduction

Aerial leaf surfaces are colonized by a variety of microorganisms including bacteria. While many of these bacteria are harmful to the plant, many more are harmless or even beneficial to the plant. Although relatively little research has been conducted on the leaf habitat compared to other microbial environments, the majority of the work has been to investigate pathogens and ice nucleating bacteria. For some pathogens, high bacterial populations on the aerial leaf surface correlate with increased disease incidence. A better understanding of the population dynamics of bacteria on the aerial leaf surface and the environmental conditions that influence these dynamics could lead to better prediction and management of foliar bacterial diseases.

Due to the very nature of the aerial leaf, bacteria located on the surface are exposed to harsh conditions, including fluctuations in UV radiation, temperature, water availability and wind. Depending on the location and/or the adaptation abilities of the bacteria, these conditions may pose a significant threat to the survival and growth of bacteria. Of the environmental conditions present on the aerial leaf surface, water deprivation may be the most important factor influencing population dynamics. Some bacteria are able to escape the leaf surface by entering into the internal leaf spaces. Although the bacterial cells may no longer encounter the harsh environmental conditions found on the surface, the cells may still be exposed to a variety of stresses due to plant responses including active oxygen species and changes in water availability. Several instruments are available that measure the water potential of soil or whole leaves; however, these instruments do not function at the scale of a bacterium. At present, the amount of water limitation bacteria encounter on the aerial leaf surface is not known. Therefore, to measure the availability of water to bacteria on and in the leaf, we proposed the use of the bacteria themselves as tools. To create these "bacterial biosensors", the water deprivation-responsive proU promoter was fused to the reporter gene, gfp, which encodes a green fluorescent protein or inaZ, which encodes an ice nucleation protein, and these fusions were introduced on a broad-host-range plasmid into a variety of strains. The water deprivation levels encountered by bacteria both on and in aerial leaves were quantified.

Thesis Organization

This thesis is divided into five chapters. The first chapter presents a review of the literature on the dynamics of bacterial populations in and on leaves, bacterial adaptation to
environmental stresses and specifically to water limitation, approaches for measuring water deprivation in the environment and the use of reporter-based bacterial biosensors for sensing environmental conditions. The second chapter is a published manuscript on the construction and characterization of our water deprivation-responsive bacterial biosensors. In third chapter, the population dynamics and water deprivation encountered by the saprophytic bacterium *Pantoea agglomerans* strain BRT98 on the surface of bean leaves is investigated. In the fourth chapter, we investigate the population dynamics and water deprivation encountered by the pathogenic bacterium *Pseudomonas syringae pv. tomato* strain DC3000 in internal spaces of *Arabidopsis thaliana* leaves. In the fifth chapter, the expression of the water deprivation-responsive transcriptional fusion in a variety of prokaryotes is explored. General conclusions are provided after chapter five. Two appendices follow the conclusions. Plasmid stability is presented in Appendix 1, while the investigation of a second label for the bacterial biosensors is presented in Appendix 2.
Chapter 1. Literature Review

Microbial Populations in the Phyllosphere

Many microorganisms including fungi, bacteria and yeast are found on aerial leaf surfaces. The most numerous of these microorganisms are bacteria, each of which functions in a distinct manner. For example, bacteria that are able to fix atmospheric nitrogen (68, 85) or produce plant growth hormones (47) on leaves may benefit plant health (65, 71, 97, 116). Others have a commensalistic relationship with the plant. Yet others are able to cause disease or induce frost injury (100, 105, 106) and are clearly potentially detrimental to plants. The phyllosphere refers to the area that is influenced by the above-ground plant structures. Generally microbes that multiply on the surfaces of plants are called epiphytes, while endophytes are generally those microbes that can multiply in internal spaces. We refer to those microbes that cause disease as pathogens and those that are harmless as saprophytes or non-pathogens. Investigations of the microbial populations in the phyllosphere have traditionally focused on plant pathogens as well as those bacteria that cause frost damage. For some pathogens, large epiphytic populations have been correlated with an increase in disease incidence and severity (16, 67, 106). Many investigators have demonstrated that environmental conditions impact the sizes of epiphytic populations (66, 68, 83, 91). Since epiphytic populations are generally the source of inoculum, it is important to understand how large bacterial populations develop on leaves. This knowledge should improve disease prediction and management.

Location of Bacteria on the Leaf Surface and Beyond

Bacteria are not distributed uniformly on the aerial leaf surface. For example, imprinting studies demonstrated that bacteria reside in particular sites (90, 92, 171). Using scanning electron microscopy, bacteria were observed to favor sites such as at the stomates (110-112, 120), base of the trichomes (12, 89, 110, 112), and epidermal cell wall junctions (20, 40, 89) especially near the veins (90, 110, 112). When researchers compared the number of bacteria located on the upper and lower leaf surfaces, more bacteria were generally present on the lower surfaces. This could be due to a higher density of stomates and trichomes, which may alter conditions in the boundary layer by increasing vapor pressure and reducing exposure to stressful conditions (25, 90, 121). It could also be due to the reduced UV exposure on lower leaf surfaces.
Although there is a strong quantitative relationship between epiphytic populations and disease occurrence for some pathogens, it is likely that bacterial pathogens must be in the internal leaf spaces to induce disease symptoms (19). Phytopathogenic populations of *Pseudomonas syringae* pv. *syringae* were shown to be primarily located within "protected sites", which included internal leaf spaces as well as external sites that protect cells from a surface sterilant (173). Several studies have shown that in the days following inoculation of various bacterial species onto leaves, only a small fraction of the inoculated organisms could be washed off (173). This finding indicates that the cells may be in sites that prevent removal. When a pathogen arrives on the aerial leaf surface it may have the ability to enter into the leaf via stomatal openings, wounds or hydathodes, and then it may access interconnected spaces within the leaf. Phytopathogenic bacteria often develop high internal populations while non-pathogenic bacteria generally do not (148, 173).

**The Leaf Habitat**

The leaf offers a variety of habitats both internal and external for bacteria. Due to fluctuations in temperature, water availability, and intensity of solar radiation, residents on the aerial leaf surface are exposed to a very harsh environment. In addition, bacteria on the leaf surface may be exposed to such toxic compounds as plant-produced hydrogen peroxide and other anti-microbial compounds (13, 15, 16). Those bacteria that are able to enter internal leaf sites may have their own set of conditions that a bacterial cell must tolerate. Some microbes may modify the internal habitat, such as by inducing plant cells to release nutrients. However, some microbes are exposed to plant defenses. In this case an elaborate cascade of plant defenses is turned on including the production of reactive oxygen species, potassium ion efflux and extensive electrolyte leakage. The bacteria, therefore, may be exposed to a harsh environment in the internal spaces as well.

Microbes may utilize at least two strategies to survive and even grow in association with leaves (16). Bacteria may tolerate environmental conditions through a series of adaptations that are discussed below. Bacteria may also avoid environmental conditions by actively moving to protected sites on or in the leaf. It has been speculated that, in general, saprophytes employ a strategy of tolerance because they survive on aerial leaf surfaces and tend to be poor colonists of internal leaf sites (149). However, a recent study provided evidence that a saprophytic bacterium, *Pseudomonas fluorescens*, entered the stomata and colonized the vascular system of apple leaves (51). Many pathogens are thought to employ
both tolerance and avoidance strategies based on their ability to establish populations on
the leaf surface as well as in internal sites. Some researchers demonstrated that a greater
proportion of pathogenic than non-pathogenic bacteria were present in protected sites (148,
173) and that pathogens established larger populations than non-pathogens following
infiltration into leaves (173).

Adaptation to Environmental Stresses

Bacteria have evolved several adaptations to tolerate or avoid environmental
stresses on leaf surfaces. For example, many bacteria that colonize the leaf produce an
extracellular polysaccharide (EPS) layer (43, 59). EPS is thought to provide protection to
the bacterial cells against desiccation and UV radiation as well as toxic compounds such as
anti-microbial products (13, 16). Some microorganisms produce carotenoids and other
pigments that may offer protection against some UV radiation (49, 153, 155). Bacteria also
often form aggregates on leaf surfaces (101, 124, 148); these may act as a protective
barrier against water deprivation, toxic compounds, and UV radiation. Motility allows
bacteria to move to sites protected from environmental stresses (57). This was
demonstrated by a report that a non-motile mutant of the phytopathogen Ps. syringae
developed smaller populations than the motile parent under dry conditions and when
exposed to UV radiation but not under moist conditions (57). Researchers have
demonstrated that UV tolerance in Ps. syringae is due to the rulAB genes located on a
plasmid (81, 82, 154). This UV-inducible locus confers DNA repair.

If a bacterial cell enters the internal leaf spaces, it may need distinct traits to adapt to
this different environment compared that on the leaf surface. The internal leaf environment
may offer several advantages to a microorganism when compared to the external
environment; these include less competition for space or nutrients, less UV radiation, more
consistent water availability and the presence of higher levels of nutrients. However, the
internal environment may also pose a unique challenge to invading microorganisms due to
the low pH (6, 7) and possible exposure to plant defenses. Phytopathogens seem to have
the ability to change the internal environment. For example, the presence of pathogens
corresponds with increased leakage of water and nutrients from the host cell (1, 24, 172).
This release of water and nutrients could support the growth and survival of the bacteria.
The presence of EPS around the bacterial cell may help reduce the impact of low water
availability because water can be retained in the hygroscopic matrix.
Various Forms of Water Availability

Exposure to limited water availability may be detrimental to microorganisms on the aerial leaf surface. Several studies have demonstrated that leaf-associated bacterial populations increased in the presence of water, but decreased under dry conditions (67, 68). Osmosensitive mutants of a *Ps. syringae* strain were able to grow to similar population levels as the wild-type under moist conditions but the mutant populations severely decreased when exposed to dry conditions (14, 17, 104). Limited water availability on the aerial leaf surface could occur by either high solute concentrations or by the lack of water. During the morning hours a thin film of water may be present, but as the temperature rises the free water present may decrease. Any solutes present on the leaf surface will accumulate in the remaining water and, therefore, the osmolarity may increase. A bacterial cell on a leaf surface may also be exposed to matric stress, or desiccation stress, if all or most of the water evaporates. Bacteria may also encounter limited water availability in internal spaces of the leaf due to accumulation of ions, such as results from to plant responses, or due to a lack of water. In general, water potential is composed of the sum of the osmotic potential, which can be controlled using solutes that can enter the cell, or the matric potential, which can be controlled using solutes that cannot enter the cell as well as adsorbing water, therefore, making it unavailable to the cell.

Mechanisms of Adaptations to Limited Water Availability by *Escherichia coli* and *Salmonella typhimurium*

The ability of microorganisms to survive and grow in a given habitat is dependent on physical parameters including the osmolarity of the environment. Bacteria need to be able to maintain an osmotic pressure in the cytoplasm that is greater than the environment. This results in outward pressure or turgor. Turgor is the necessary force for cell expansion during growth. If a cell is exposed to an osmotic upshift, a rapid efflux of water from the cytoplasm occurs. The efflux of water causes turgor loss, plasmolysis and dehydration. To restore turgor, bacteria accumulate potassium ions. Compatible solutes such as glycine betaine, trehalose and proline are synthesized or taken up without disturbing cellular processes. Compatible solutes stabilize cell components and protect proteins from denaturation (5). Transport systems are used by the cell to accumulate solutes. If a cell is exposed to an osmotic downshift, a rapid influx of water from the environment occurs causing an elevation in turgor. The cytoplasmic membrane and cell wall could be ruptured if
the turgor pressure becomes too high; this results in cell death. To prevent lysis, mechanosensitive channels are activated by the cell and solutes exit the cell.

It has been proposed that bacteria respond to osmotic upshifts and downshifts in 3 overlapping phases (174). The following is an overview of these phases, each of which will be discussed in more detail below, in *E. coli* cells. The phases of an osmotic upshift are dehydration (phase 1), adjustment of cytoplasmic solute concentration and rehydration (phase 2), and cellular remodeling such as the cell wall and nucleoid changes (phase 3). These phases have been well studied. Phase 1 occurs within 1 to 2 minutes of an upshift. The cell dehydrates and shrinks, cytoplasmic crowding occurs, and respiration and most transport stops. The osmoresponsive system, ProP, is activated within the cell. Phase 2 occurs between 20 and 60 minutes and rehydration begins because K\(^+\) is first taken up by the Trk system and then glutamate accumulates as a counter ion to K\(^+\). The increase in osmolarity resulting from solute uptake or synthesis drives rehydration. Respiration also resumes but at a reduced rate. Finally, after an hour in phase 3, the cell wall and nucleoid are returned to normal, DNA and protein synthesis resumes, and cell growth and division resumes. In the course of these 3 phases, the osmoresponsive operons *proP*, *proU*, *kdpFABC* and *betT* are expressed. The phases of an osmotic downshift are uptake of water (phase 1), release of water and solutes (phase 2), and solute reaccumulation and cellular remodeling (phase 3) (174). Phase 1 occurs within a minute of an osmotic downshift, the bacterial cell swells causing a decrease in cytoplasmic crowding, and mechanosensitive channels are opened. During phase 2 (1-2 minutes), the cell shrinks, crowding increases, and solutes and water are released. Phase 3 occurs between 10 to 20 minutes and channels close and solutes re-accumulate (174). These responses to an osmotic downshift have not been investigated thoroughly.

In addition to the major K\(^+\) uptake system, Trk, in *E. coli*, a second major K\(^+\) uptake system, Kdp, and a minor one, Kup, are induced. Homologs of these systems have been found in *S. typhimurium* and other bacteria (132). The Trk proteins have a modest affinity for K\(^+\) and are constitutively expressed (9). The influx of K\(^+\) via the Trk system is equal to the efflux in non-stressed exponentially growing cells. The Kdp system is transiently inducible to a relatively high rate (>1000-fold) (130), but has a high affinity for K\(^+\) (2). When potassium ions are present at low concentrations, the cell uses this system to scavenge. Finally, the Kup system is a minor uptake system compared to Trk and Kdp. It has a modest rate of uptake and affinity to K\(^+\) and it is only induced approximately 2-fold (143).
One of the most common responses to changes in environmental osmolarity is the accumulation of compatible solutes, which protect cells and allow for growth. K\(^+\) and glutamate inhibit many enzymes at high concentrations (144). The cell replaces ions with compatible solutes reducing the negative effects of ions on enzymatic reactions (29). Compatible solutes are osmotically active solutes that can be accumulated or synthesized to high concentrations in the cell without disturbing metabolic functions. Compatible solutes are thought to increase the free water content of the cell and cytoplasmic volume (29, 142). In addition, compatible solutes are thought to stabilize proteins (5, 29) although this process is not completely understood. Based on the preferential exclusion model, hydration of the proteins is thought to occur due to the presence of compatible solutes. Trehalose is a compatible solute that is accumulated by the cell when exposed to either osmotic or matric stress (34, 164). Some other examples of common compatible solutes are glycine betaine, proline betaine, proline and ectoine. Microorganisms have a tendency to utilize one compatible solute over another if it is available in the environment. For example, *E. coli* and *S. typhimurium* prefer betaine over proline betaine over proline (135). Very little is known about cell responses to matric stress, therefore, the following discussion focuses on osmotic stress.

ProP and ProU are osmotically regulated permeases that help in the uptake of most compatible solutes in *E. coli* and *S. typhimurium*. Originally the two systems were shown to transport proline (4, 35, 44), but later they were also found to transport betaine and other compatible solutes (11, 26, 27). The ProP permease has similar affinities for transporting proline, betaine and ectoine (76, 175). The promoter seems to have a slower rate of acceleration when compared to K\(^+\) uptake by the Kdp promoter, and is usually induced between 2- and 5-fold (122, 143). The ProU permeases have a high affinity for betaine but a low affinity for both proline and ectoine (26, 76). ProU permeases, a high-affinity transport system, is a member of the ATP-Binding Cassette (ABC) superfamily of transporters (60). The *proU* operon is composed of three genes encoding two cytoplasmic membrane-bound proteins, ProV and ProW, and the periplasmic binding protein ProX (11, 38, 56, 62) collectively known as the ProU permeases. The *proU* operon is induced greater than 100-fold by an osmotic upshock, which is higher than the *proP* promoter but not as high as the *kdp* promoter (26, 44, 55). As long as the cell remains under the osmotic stress, the *proU* operon is expressed.
Although the mechanisms for adaptation to osmotic upshift have been well studied in *E. coli* and *S. typhimurium*, the same is not true for other microorganisms. However, some systems have been identified. For example, *Bacillus subtilis* contains OpuA and OpuC that are specific to the uptake of glycine betaine (79, 80). Also many microorganisms have structurally and functionally similar transporters called the Bet system for taking up betaine, choline and carnitine. The Bet system is not only found in *E. coli*, but also in *Listeria monocytogenes, Mycobacterium tuberculosis, Sinorhizobium meliloti* and *Corynebacterium glutamicum* (46, 88, 136-138, 151, 152). Finally, many organisms including *E. coli, S. meliloti, Erwinia chrysanthemi* and *Ps. aeurginosa* use glycine betaine as a compatible solute (53, 133, 157).

**Effect of Limited Water Availability on Growth and Culturability**

Most researchers in this field have investigated bacterial tolerance to osmotic shock. In general, an increase in osmolarity may not kill the microorganisms, but instead may stop growth (147). Jorgensen *et al.* (1994) observed that 1 M NaCl reduced culturability of *Ps. fluorescens* by 1 log unit, but 60% were still viable, while 1.7 M NaCl reduced culturability by 4 log units, but 50% were still viable (78). In a similar study, 0.7 M NaCl did not have any effect on colony forming ability of *Ps. fluorescens*; however, at 1.5 M NaCl the number of culturable cells decreased by 3 log units (113). For *E. coli* cultures, the addition of 0.8 M NaCl to medium left 90% of the cells viable but not culturable (147). A slow decrease in water potential had less of an impact on cell physiology than a more rapid decrease in water potential. For example, a slow water potential decrease resulted in 80-100% culturability in three bacterial species whereas a rapid decrease resulted in 15-57% culturability (139). Also, large osmotic upshifts caused the loss of motility followed by pseudotumbling in *E. coli* (94), a change in cell size (10), and the immediate and transient inhibition of respiration (119). In addition to loss of culturability, a reduction in growth rates and the increase in lag time occurred with the addition of NaCl. For example, the addition of 0.4 M NaCl to a liquid culture of *Erwinia chrysanthemi* reduced the rate of growth between 4 and 12 h after inoculation (160). Similarly, the doubling time increased from 50 minutes to 230 minutes when *E. coli* cultures grown with 0.6 M NaCl were exposed to 3 M NaCl (118). In addition, the researchers observed that the lag time increased linearly with increased osmolarity (118). Holden *et al.* (1997) demonstrated that cultures had better growth rates with a water
potential of \(-0.25 \text{ MPa}\) due to the addition of PEG8000 than without addition of PEG8000 (70).

**Water Potential Measurement**

At least four distinct instruments have been developed to evaluate the water potential of an environment: an osmometer, a thermocouple psychrometer, a wetness sensor and a dew point hygrometer. An osmometer is restricted to measurements in liquid media and therefore cannot be utilized to measure leaf wetness. A thermocouple psychrometer can determine the water potentials of excised leaves (23, 28, 84, 176). To use this instrument, for each sample a water vapor pressure equilibrium between the sample and the air in the chamber must be established. The waxy cuticle of the leaf causes slow equilibration of the vapor pressure within the instrument. The thicker the waxy layer the longer the equilibration period. Researchers have reported that excised leaf material dries out during this equilibration period. One researcher observed that leaf water potentials became progressively lower with increasing times in a controlled environment (23). In addition, the instrument must be calibrated with samples of various salt concentrations and readings must be corrected using the temperature of the environment. Wetness sensors have been used in field experiments to measure leaf wetness duration (48, 140). These sensors give readings indicating merely wet or dry. Wetness sensors are very sensitive to the placement of the sensor (140). Wetness sensors also need to be properly calibrated. Finally, a dew point hygrometer can measure the water potential of solutions, soils and excised plant tissues (125). A sample is placed into the central chamber and air is blown through tubes at the base to cool the chamber. Condensation forms in the chamber, temperatures of the two thermometers are read and humidity is determined using standard tables. In one study, a hygrometer gave similar readings to a thermocouple psychrometer for one leaf type but not for another (125). However, since equilibration of the samples for a hygrometer ranged from 3 h for soybean leaves and 6 to 8 h for cereal leaves, the water potential of the leaves may have changed during the equilibration period (125). Although many of these sensors can provide estimates of the water potential of leaves, these estimates may not reflect the water potentials sensed by bacteria on those leaves. Importantly, these instruments do not function at the scale of a bacterium. The ideal tools to measure water availability in microbial habitats are the bacteria themselves. Therefore, we proposed to design bacterial
biosensors that could be used to evaluate the availability of water to bacteria on and in leaves.

**Bacterial Biosensors to Monitor Abiotic Environmental Conditions**

There are a variety of examples of the successful use of genetically-engineered bacteria to measure environmental conditions encountered by bacteria in their microhabitat. For example, an arcDABC-lacZ-based *Ps. fluorescens* biosensor was used to monitor oxygen availability in the soil habitat (69). A katG::lux fusion in E. coli was used to detect oxidative stress in culture (18), while a recA::lux fusion in *Ps. aeruginosa* was used to study the effects of UV radiation on a biofilm bacterial community (45). The green fluorescent protein (GFP) was fused to three promoters, rpoH (for $\sigma^{32}$), dnaK and clpB, to detect *E. coli* cellular stress responses in cultures to heat shock, nutrient limitation, and ethanol addition (30). Some examples of bacterial biosensors used on plant surfaces include a scrY- and aatl-inaz-based *Erwinia herbicola* biosensor used to map the availability of both sugar and amino acids in soil near the roots (75) and a fruB-gfp-based biosensor to test for sugar consumption by *E. herbicola* in the phyllosphere (93). Bacterial biosensors have also been used to detect pollutants such as polychlorinated biphenyls in the environment (87).

Reporter genes are critical to the function of bacterial biosensors. Many reporter genes have been used to monitor gene expression in microorganisms. Some of the most widely used reporter genes have been lacZ, phoA, luxAB, inaz and gfp. There are advantages and disadvantages of each. The $\beta$-galactosidase gene, lacZ, is a commonly used reporter gene because the activity can be easily measured after the addition of either a chromogenic substrate (5-bromo-4-chloro-3-indolyl-$\beta$-D-galactopyranoside) or a fluorogenic substrate (fluorescein di-D-galactopyranoside). The use of lacZ to monitor gene expression in the phyllosphere is limited due to the high background levels of indigenous $\beta$-galactosidase-producing prokaryotes and eukaryotes (103). *E. coli* alkaline phosphatase encoded by phoA has a location-sensitive activity. It is useful to identify only periplasmic or membrane-associated proteins because PhoA is not active in the cytoplasm. The bioluminescence operon (luxAB) can also be used to monitor real-time gene expression. This operon encodes the luciferase enzyme, which is necessary for light generation in *Vibrio fischeri*. This reporter system is dependent on oxygen, reduced flavin mononucleotide (FMNH$_2$) and an aldehyde substrate, which can be added exogenously or synthesized by using luxABCDE as a reporter (117). The usefulness of luxAB as a reporter is restricted to
metabolically active cells. Similar to other reporter genes the \textit{inaZ} reporter gene can be used to monitor gene expression in a population of cells, although not in an individual cell. The \textit{inaZ} gene encodes a protein that is capable of nucleating the formation of ice. Ice nucleation activity measurements can be very time-consuming, temperature sensitive and have a rapid turnover rate, which may or may not be a limitation. The \textit{uidA} gene, which encodes the Gus protein, in \textit{E. coli}. The gene produces the enzyme \textit{\beta}-glucuronidase, which cleaves 5-bromo-4-chloro-3-indoyl-1-glucuronide and produces a colored product (77). The product can be easily and inexpensively analyzed using fluorometers and spectrophotometers, although these require destructive sampling, as well as visualized using light microscopy. Since this system uses enzymatic action, it can be amplified and easily detected (145). The enzyme is also very stable. Another reporter gene is \textit{xylE}, which encodes catechol-2,3-oxygenase. This enzyme converts catechol to 2-hydroxymuconic semialdehyde, which is a bright yellow pigment that can be visualized or measured spectrophotometrically (181). This gene originates from \textit{Ps. putida} (50). The stability of the enzyme varies with oxygen availability and the physiological state of the cell (86). Use of this reporter gene is, therefore, best in exponentially-growing cells. The enzyme is also inhibited by reactive oxygen species (86). The \textit{gfp} reporter gene has several advantages including ease of detection of gene expression at a cell and population using flow cytometry. The protein is very stable, requires oxygen and has at least a 2-h incubation time for proper folding to occur. In addition, no co-factors are required.

\textbf{Construction of a Water-Responsive Bacterial Biosensor}

The construction of a bacterial biosensor requires a fusion between two components: a promoter that responds to the desired environmental signal and a reporter gene that is easily detected. Therefore, for the water-responsive bacterial biosensor, the \textit{P}_{\text{proU}} from \textit{E. coli} was used with either the \textit{gfp} gene or the \textit{inaZ} gene. The \textit{proU} operon encodes a transport system for the uptake of various compatible solutes. Both components of this bacterial biosensor will be discussed in greater detail below.

\textbf{The \textit{proU} Promoter}

There are two promoters driving the \textit{proU} operon. The major one, P2, is recognized by the RpoD-RNA polymerase holoenzyme (179), while the minor promoter P1, which is located 189 bp upstream from P2, is recognized by the RpoS-RNA polymerase (39, 109). A
negative transcriptional regulatory element is located between 75 and 300 bp downstream of P2 within the proV gene; this element acts as a silencer (39, 129). Deleting this downstream regulatory element increases the basal level of expression of the proU operon by 40-fold.

Despite years of research to identify regulatory proteins necessary for osmotic control of the proU operon, no putative regulators have been found. Numerous possibilities have been suggested for the control of this operon. In one model, elevated K\(^+\)-glutamate concentrations were thought to directly initiate P\(_{proU}\) transcription (61, 63, 72, 161). However, activation by K\(^+\)-glutamate appears to be a nonspecific effect because K\(^+\)-glutamate initiates transcription of genes that are not osmotically controlled (36), and increasing glutamate concentrations \textit{in vivo} were not necessary for osmotic induction of the P\(_{proU}\). Others have proposed that the H-NS protein may regulate P\(_{proU}\) expression because the protein has a high affinity to curved DNA (158) and binds upstream (162) and downstream (108) of the proU promoter. The H-NS protein was found to compete with the RNA polymerase for binding to the proU promoter \textit{in vitro} (39, 108, 109, 131). Although \textit{hns} mutants were reduced in both basal and induced levels of P\(_{proU}\) expression, they did not influence the osmotic induction of the system (109). Therefore, H-NS is not responsible for the osmoresponsiveness of the proU operon. In the final model P\(_{proU}\) expression has been proposed to be regulated by a change in the topology of the DNA. Specifically, the topology change may alter the downstream regulatory element along with the proU promoter and the H-NS protein (131, 159, 177). This model is supported by three lines of evidence: DNA supercoiling changes in response to high or low osmolarity (131, 134, 163, 180), H-NS changes the topology of DNA \textit{in vitro} (26), and no other regulatory proteins have been identified (55, 114, 115, 131).

Characterization and Use of the Green Fluorescent Protein

The green fluorescent protein (GFP) originates from various coelenterates including \textit{Aequorea}, \textit{Obelia} and \textit{Renilla} (123, 165); however, only the \textit{Aequorea} and \textit{Renilla} GFP have been well characterized (168). The commonly used GFP originated from the jellyfish \textit{Aequorea victoria} and was discovered by Shimomura et al (150). The green fluorescence emitted from the jellyfish was due to GFP converting the blue emission of aequorin into the green glow (123, 150, 167). Cloning of the \textit{gfp} gene by Prasher et al (141) and the demonstration that expression of the gene results in visible fluorescence in other organisms
(31, 73) demonstrated that GFP did not require any co-factors or jellyfish-specific enzymes; this enabled its widespread use.

The GFP consists of 238 amino acids, but only amino acids 7-229 are needed for fluorescence (95). The GFP chromophore is comprised of the three amino acids Ser<sub>65</sub>-Tyr<sub>66</sub>-Gly<sub>67</sub> (32). The chromophore is surrounded by an α-helix that is tightly packed in a β-can structure (127, 178). The chromophore formation occurs post-translationally through a series of steps including a cyclization reaction and an oxidation step that requires oxygen (41, 58). Chromophore formation is thought to be the rate-limiting step and can take at least 2 h (33, 37, 58).

Wild-type GFP (wt GFP) absorbs UV light at a maximum absorbance of 395 nm and emits light maximally at 509 nm (167). Many GFP derivatives have been created and used by altering one of more of the three amino acids that make up the chromophore. This alteration changes the excitation and/or emission maximum of the derivative. For example, the widely used red-shifted GFP variant contains a mutation in amino acid 65 from Ser to Thr, which changes the maximum excitation from 395 nm to 489 nm without affecting the emission maximum. Similarly, the blue fluorescent protein, which has a maximum excitation of 384 nm and maximum emission of 448 nm, was created by changing the amino acid at 66 from tyrosine to histidine.

The wt GFP, enhanced GFP (EGFP), and red-shifted GFP fluorescence is very stable in a fluorometer and resistant to photobleaching, irreversible damage of the chromophore, when illuminated by 450-490 nm light. However, some photobleaching can occur when illumination of 340 to 440 nm is used on wt GFP (31, 126). Weak reducing agents such as 10 mM dithiothreitol (DTT) or moderate oxidizing agents do not affect GFP fluorescence; however, strong reducing agents such as Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> do (74). GFP is stable in the range of pH 5.5-12, but fluorescence intensity decreases if the pH drops below pH 5.5 or rises above 12. GFP is resistant to heat, detergents and most proteases (22, 166). GFP also has been shown to be very stable, having a half-life of greater than 24 h in various organisms (96). Unstable variants of GFP have been constructed to increase turnover time for measuring changes in gene expression (3). Finally, although GFP has been shown to be heat stable, the formation of the chromophore is temperature sensitive. For example, GFP fluorescence is more intense when grown at 24°C or 30°C compared to 37°C (58).

GFP is a very powerful and versatile reporter system to investigate protein localization or gene expression in both prokaryotes and eukaryotes. For example, GFP has
been used to study host-pathogen interactions. It is also useful to constitutively tag microorganisms and monitor them in a given habitat. For example, *Pseudomonas* spp. were tagged with GFP and monitored near the roots (21). Major organelles of the cell such as nuclei (98, 145) and the mitochondria (42, 145) have been successfully targeted by GFP or GFP derivatives. In fact, researchers have been able to label each organelle with a distinct GFP derivative allowing for multi-color visualization of the cells. The use of GFP and its derivatives has allowed and will continue to permit the exploration of many areas of prokaryotic and eukaryotic biology.

**Characterization and Use of the Ice Nucleation Protein**

Many materials including organic and inorganic substances can catalyze ice formation. There are a number of bacterial and fungal species that produce biological ice nuclei, with the best characterized being *Pseudomonas syringae*. The ice nucleation activity in *Ps. syringae* is conferred by a single gene *inaZ* that encodes an outer membrane protein (170). Ice nucleation genes vary in size (3.2 to 5 kb) among species. A single ice nucleation protein cannot act as an ice nucleus, but rather the individual proteins need to form aggregates that are able to align water molecules and catalyze ice formation (169). The warmest temperature a sample can be cooled to before ice nucleation occurs is proportional to the logarithm of the number of ice nuclei in the sample (64, 102, 156). Activity at warmer temperatures requires larger aggregates of the ice protein to be present (54). Cells containing ice proteins are not all active at the same temperature; in fact, activity can occur between −2 and −10°C (54). This reporter gene is advantageous for a variety of reasons. First, the gene can be used in a variety of microorganisms. For example, this gene has been moved to the organisms *E. coli*, *Pseudomonas aureofaciens*, *Erwinia amylovora*, *Xanthomonas campestris*, *Rhizobium meliloti* and *Agrobacterium tumefaciens* and shown to confer ice nucleation activity (52, 99, 103, 107, 128). The *inaZ* gene has also been used successfully in plants (8). The *inaZ* gene is very sensitive and has a wide range of activity. It can be used in a wide variety of environments including soil, water and the rhizosphere due to very low levels of background ice nucleation activity. Ice nucleation can be detected in environmental samples such as soil slurries in which other reporter gene assay would encounter interference (107). The protein also has a rapid turnover rate, which is useful for some application such as detecting repression of gene expression. There are also disadvantages to this system. The droplet freezing method for the quantification of ice
activity is time consuming and temperature sensitive. Samples are collected and diluted and drops of each sample are tested for freezing. Some cells retain the ice nucleation activity after viability is lost; therefore, droplets containing non-viable cells will still freeze.

Although both reporter genes gfp and inaZ have limitations in their use, the advantages of each make them appealing to use as reporter systems for our purposes. Analysis of GFP is fast and cost effective. In addition, a great deal of information including cell size and inclusions can be obtained by analyzing GFP-labeled cells on the flow cytometer. However, in some applications on aerial leaves some GFP-labeled cells can not be distinguished from plant debris. The use of the inaZ reporter gene does not have that limitation. InaZ can be detected and quantified over a 20,000-fold range of expression. We are interested in determining the actual water availability bacteria encounter on and in aerial leaves. To do this, we have created bacterial biosensors that produce either GFP or InaZ in response to water deprivation.

References


100. Lindow, S. E. 1987. Competitive exclusion of epiphytic bacteria by ice


Chapter 2. Construction and Characterization of a proU-gfp Transcriptional Fusion that Measures Water Availability in a Microbial Habitat

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Abstract

We constructed and characterized a transcriptional fusion that measures the availability of water to a bacterial cell. This fusion between the proU promoter from *Escherichia coli* and the reporter gene *gfp* was introduced into strains of *E. coli*, *Pantoea agglomerans*, and *Pseudomonas syringae*. The proU-gfp fusion in these bacterial biosensor strains responded in a quantitative manner to water deprivation caused by the presence of NaCl, Na₂SO₄, KCl, or polyethylene glycol (mol. wt. 8000). The fusion was induced to a detectable level by NaCl concentrations as low as 10 mM in all three bacterial species. Water deprivation induced proU-gfp expression in both planktonic and surface-associated cells; however, it induced a higher level of expression in the surface-associated cells. Following the introduction of *P. agglomerans* biosensor cells onto bean leaves, the cells detected a significant decrease in water availability within only 5 min. After 30 min, the populations were exposed, on average, to a water potential equivalent to that imposed by approximately 55 mM NaCl. These results demonstrate the effectiveness of a proU-gfp-based biosensor for evaluating water availability on leaves. Furthermore, the inducibility of proU-gfp in multiple bacterial species illustrates the potential for tailoring proU-gfp-based biosensors to specific habitats.

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Introduction

A key issue in microbial ecology is understanding how the abiotic environment affects microbial populations. In terrestrial habitats, microorganisms reside in microsites that may vary greatly in their abiotic conditions. Instruments that measure environmental parameters, such as pH, water availability, and solar radiation, typically do not function on the scale of a microorganism. Tools that measure abiotic conditions on a microscale would be invaluable for understanding the conditions that microbes actually encounter in their native habitats, and thus for predicting the impact of these conditions on their growth, survival, and physiological state.

Our goal was to create a tool that will enable microscale measurements of water availability in terrestrial habitats. Water availability is clearly critical to the physiological state of microorganisms, and it may be a key factor influencing bacterial growth and survival in many habitats, including in soil and on the surfaces of plants and animals. Previous studies have demonstrated that bacteria containing transcriptional fusions between an environmentally-responsive promoter and a reporter gene can effectively report on the abiotic conditions sensed by a microorganism. Examples include a lacZ-based Pseudomonas fluorescens biosensor that was used to quantify oxygen availability to bacteria in soil (20), a lux-based Pseudomonas aeruginosa biosensor that was used to quantify bacterial exposure to UV radiation in biofilms (15), and gfp-based biosensors that were used to monitor sugar consumption by bacteria on leaves (27) and roots (8). Similar biosensors have been constructed that detect pollutants and toxic chemicals (see, e.g., (44, 45, 48, 49)), as well as oxidative stress, heat shock, and nutrient limitation (4, 12).

The promoter of the proU operon (P_{proU}) in Escherichia coli is ideal for use in a whole cell bacterial biosensor to detect water availability, because it responds rapidly to low water potentials and it maintains a high level of expression as long as the cells remain under a low water potential. The function of the proU operon is to aid in osmoadaptation by encoding a transport system for the uptake of various compatible solutes, including glycine betaine and proline. Previous studies have demonstrated that P_{proU} expression in E. coli and Salmonella typhimurium is proportional to the osmolarity of the growth medium (19, 31, 34). Previous studies have also shown that P_{proU} exhibits osmoregulation without a regulatory protein (32, 33), indicating that it may be osmoregulated when a simple P_{proU}-reporter gene fusion is introduced into other bacterial species.
The use of the reporter gene gfp allows reporter activity to be examined in individual cells. Because the reporter activity is cellular fluorescence conferred by the production of a green fluorescent protein (GFP) (50), fluorescence microscopy can be used to generate spatial information of biosensor cells in situ, and flow cytometry can be used to quantify the fluorescence of individual cells recovered from a habitat. Additionally, unlike the reporters β-galactosidase, β-glucuronidase, and ice nucleation proteins (28), GFP is not known to be produced by organisms indigenous to terrestrial habitats; thus, it can be used without background reporter activity by the indigenous microflora. GFP is very stable (42); this provides experimental flexibility when evaluating reporter activity following an induction event, although studies focused on the dynamics of promoter expression over time require the use of short-lived GFP variants (1). The primary disadvantage of GFP as a reporter protein is the delay between protein production and protein fluorescence; thus, rapid induction events can only be examined if conditions can be imposed that prevent further promoter activity during a post-induction incubation period.

The objectives of this study were to construct transcriptional fusion-based biosensors that respond to water deprivation in a quantitative manner, to identify the effect of bacterial species and bacterial growth conditions on this quantitative response, and to test the biosensors for their ability to detect bacterial exposure to water deprivation in one terrestrial habitat, on a leaf surface. For these studies, we introduced a proU-gfp fusion into three bacterial species, E. coli, Pantoea agglomerans, and Pseudomonas syringae. The latter two are commonly found on plant leaves. We present evidence that the proU-gfp fusion responds quantitatively to water deprivation in both planktonic and surface-associated cells of all three bacterial species, and that a bacterial strain containing the proU-gfp fusion was an effective tool for evaluating the availability of water to bacterial cells on plant leaves.

Materials and Methods

Bacterial strains and growth conditions.

The bacterial strains used in this study included Escherichia coli strain DH5α (Gibco-BRL, Rockville, MD), Pseudomonas syringae pv. syringae strain B728a (30), and Pantoea agglomerans strain BRT98 (3). Strains B728a and BRT98 were resistant to rifampin, and the plasmids that were used (described below) conferred resistance to kanamycin. The strains were grown in LB medium (37) or in the low osmoticum medium one-half strength 21C (½-21C) (16, 46). The ½-21C medium was supplemented with vitamin B₁ (0.0005%)
for the growth of *E. coli*, and was amended with NaCl, KCl, Na$_2$SO$_4$ or polyethylene glycol mol. wt. 8000 (PEG 8000) to lower the water potential. The antibiotics rifampin and kanamycin were each used at a concentration of 50μg/ml. Cycloheximide (100μg/ml) was used to eliminate the growth of fungi on plates.

**Plasmids.**

Plasmid pOSEX4 (19), which was the source of the proU promoter, was obtained from E. Bremer. The broad-host-range cloning vector pPROBE-KT' (38), which contained a promoterless *gfp* gene that encoded a S65T, F64L derivative of GFP (39, 50), and the plasmid pGreenTIR (39), which contained a *gfp* cloning cassette, were obtained from W. G. Miller and S. E. Lindow. The broad-host-range cloning vector pVSP61 (22, 29, 51) was obtained from J. E. Loper.

To construct pVProGreen, the broad-host-range plasmid pVSP61 was first altered by deleting an approximately 850-bp HindIII-PvuII fragment containing 350 bp of the lacZ promoter. Next, the *gfp* gene on the plasmid pGreenTIR was amplified using the primers 5'-CGAGCTCGAATTCT and 5'-GAGCTCGAATTCC-3', and a 752-bp EcoRI fragment from the amplified product was cloned into the ΔP$_{lacZ}$ derivative of pVSP61 to form pV'Green. The *proU* promoter was amplified from *E. coli* DH5α using the primers 5'-GTCTAGTCAGACAGCCCTTATAC-3' and 5'-ACATACGTCGACATCTCGCCTTCTTC-3', in which the underlined bases indicate introduced SalI sites. A 337-bp SalI fragment containing P$_{proU}$ was inserted into pV'Green to create a proU-*gfp* transcriptional fusion, and the resulting plasmid was designated pVProGreen.

To construct pVLacGreen, the *gfp* gene on pGreenTIR was amplified using the primers 5'-CGAGCTCGAATTCT and 5'-GAGCTCGAATTCC-3', and a 752-bp EcoRI fragment from the amplified product was cloned into pVSP61 adjacent to the existing lacZ promoter (P$_{lac}$), creating P$_{lac}$-*gfp*. The plasmid pPNptGreen was constructed by PCR amplification of the *nptII* promoter (P$_{nptII}$) in Tn5 (5, 35) with the upstream primer 5'-ACTACTGTCGACGTCAGGCTGTTAC and the downstream primer 5'-GCATACGTCGACATCTCTGCTCTTG; the underlined bases indicate introduced SalI sites. A 365-bp SalI fragment containing the *nptII* promoter was inserted into pPROBE-KT creating P$_{nptII}$-*gfp*. Plasmids were mobilized into strains DH5α, BRT98 and B728a by triparental matings with the helper plasmid pRK2073 (6). The plasmid pPProGreen was constructed as described in the results.
Plant inoculation.

The inocula for the plant studies were prepared by growing the strains on ½-21C plates for 48 h at 28°C. The cells were resuspended in ½-21C to a density of $10^9$ cells/ml for experiments involving bacterial recovery from leaves and flow cytometry, and to a density of $10^7$ cells/ml for experiments involving microscopy of bacteria on leaves. Cell concentrations were determined turbidimetrically using standard curves relating cell number to optical density at 600 nm (OD$_{600}$) for each strain. Seven bean seeds were planted in 11.5-cm pots containing a 1:2:1 peat:perlite:soil potting mixture. Bean plants (Phaseolus vulgaris L. cv. Bush Blue Lake 274) were grown in a growth chamber at 45% relative humidity and 28°C with a 12-h photoperiod of 350 µeinsteins m$^{-2}$sec$^{-1}$. When the primary leaves were fully expanded, the plants were inoculated by submerging the leaves in bacterial cell suspensions.

Detection of GFP-expressing bacteria in planta.

For microscopy experiments, plants were inoculated with cell suspensions, covered with moist bags for 24 h, and then exposed to 4- and 8-h drying periods on the laboratory bench. During the exposure to drying conditions, the leaf surface wetness visibly decreased. Three primary leaves representing each treatment were sampled. Each leaf was cut from tip to base on one side of the mid-vein, and each half was divided into thirds. The adaxial surface of three sections and the abaxial surface of three sections were examined using a Leica Model DMIRB/E microscope equipped for epifluorescence. A filter set with an excitation of 450 to 490 nm and emission of ≥520 nm was used to visualize GFP production in P. agglomerans BRT98 cells on leaves. A filter set with an excitation of 340 to 380 nm and an emission of ≥430 nm was used to visualize GFP production in Ps. syringae B728a cells on leaves; this set was found to enhance detection of the low level of GFP production that occurred in strain B728a. Images were captured using a 35-mm camera.

GFP quantification.

The fluorescence of GFP-producing cells that were grown in culture was quantified by two methods, fluorometry and flow cytometry. Two fluorometers were used: a Quantech Fluorometer (Thermolyne-Barnstead, Dubuque, IA) with an excitation filter of 480±1 nm and an emission filter of ≥515 nm, and a FluoroMax2 (Jobin-Yvon-Horiba, Edison, NJ), which was equipped with DataMax software (Jobin-Yvon-Horiba, Edison, NJ) and a 150W Xenon
lamp. GFP fluorescence was measured with the FluoroMax2 using an excitation wavelength of 488±1 nm and an emission wavelength of 510±1 nm. Before analysis by fluorometry, the OD$_{600}$ of all cultures was adjusted to 0.05.

A Beckman-Coulter (Fullerton, CA) EPICS-XL-MCL flow cytometer equipped with a 15-mW, 488-nm, air-cooled argon ion laser was used in this study. GFP fluorescence was collected using a 550 nm dichroic long-pass filter and a 525±20 nm bandpass filter. Voltages were set at 860V for the GFP fluorescence detector, 790V for side scatter, and 869V for forward scatter. Fluorescence, side scatter and forward scatter data were collected with logarithmic amplifiers. GFP-expressing bacterial cells were detected based on fluorescence, forward scatter, and side scatter signals above baseline thresholds. Data acquisition was done with Coulter XL System II (version 2.1) (Beckman-Coulter, Miami, FL). Data analysis and graphical representation were done with FCS Express (version 1.0) (URL http://denovosoftware.com) software. At least 25,000 cells were examined for each sample. The geometric mean of the fluorescence and the forward scatter were calculated for all samples; these geometric means are referred to as the mean fluorescence and the mean forward scatter throughout this report. Fluorescence was expressed as relative fluorescence units (rfu).

**Characterization of P$_{proU}$-gfp induction in culture.**

The rate of P$_{proU}$-gfp induction in liquid culture was evaluated by measuring the fluorescence of a culture at various times following the addition of NaCl to final concentrations of 0 to 500 mM (0 to -2.4 MPa) (17). The effect of NaCl concentration on P$_{proU}$-gfp expression and on culturability was assessed using cells from both 24-h broth cultures and 48-h plate-grown cultures containing 0 to 1 M NaCl. The effect of the concentration of PEG 8000 on P$_{proU}$-gfp expression was assessed using suspensions of cells from a solid medium containing PEG 8000 at concentrations of 0, 15, 26, and 33%, which conferred water potentials of 0, -0.5, -1.0, and -1.5 MPa, respectively (16). The effect of distinct solutes on P$_{proU}$-gfp expression were compared using 24-h broth cultures containing various NaCl, KCl, or Na$_2$SO$_4$ concentrations to confer water potentials of 0, -1, and -2 MPa. The concentrations of NaCl required to confer these water potentials were 0, 0.22 M, and 0.439 M, respectively; those of KCl were 0, 0.221 M, and 0.449 M, respectively; and those of Na$_2$SO$_4$ were 0, 0.171 M, and 0.381 M, respectively (17).
Characterization of $P_{proU}$-gfp induction in planta.

Plants were inoculated with cell suspensions, as described above, and were immediately placed on the laboratory bench to promote evaporation of the free water on the leaves. After these drying periods, the plants were enclosed in pre-moistened plastic tents for 2 h to prevent further drying and to provide the time required for newly translated GFP molecules to assume the proper conformation for fluorescence (50). After the 2-h incubation period, three primary leaves were removed for each treatment and were each placed in 20 ml of $\frac{1}{2}$-21C broth. The tubes were sonicated gently for 7 min to aid in bacterial recovery (40). The cells in a 50 $\mu$l aliquot of the leaf sonicate were enumerated by plate count, and the remaining cells were concentrated by collection on a membrane filter (0.22 $\mu$m) followed by suspension in 3 ml of $\frac{1}{2}$-21C. Samples were analyzed using flow cytometry as described above.

Results

Construction of plasmids pPProGreen, pVLacGreen and pPNptGreen.

The $proU$ promoter ($P_{proU}$) from the pOSEX4 vector was cloned as a 612-bp EcoRI-BamHI fragment into the vector pPROBE-KT upstream from the gfp gene, creating a $P_{proU}$-gfp transcriptional fusion; this plasmid was designated pPProGreen. The $P_{proU}$-gfp fusion was flanked by T1 transcriptional terminators from the E. coli $rrnBI$ operon to prevent read-through transcription from other promoters on the plasmid (Fig. 1). The $P_{proU}$-gfp fusion contained two regions of the $proU$ promoter that were necessary for maximum osmoregulated expression, an upstream activating region that allows for maximum inducibility (32) and a downstream silencer that represses basal expression in low osmolarity media (13) (Fig. 2). Originally, a 331-bp region of the $proU$ promoter (Fig. 2B) was fused to gfp to form the plasmid pVProGreen (41). Compared to pPProGreen (Fig. 2A), pVProGreen was lacking both 138 bp of the UAR of the $proU$ promoter and 143 bp of the $proV$ gene. DH5$\alpha$ (pVProGreen) and DH5$\alpha$ (pPProGreen) exhibited similar levels of fluorescence when grown in the absence of NaCl (Table 1), and thus both cloned $proU$ promoters appeared to contain the silencer region. This supports a previous finding that the silencer region is within the first 200 bp downstream of the transcriptional start site (32). Exposure of DH5$\alpha$ (pVProGreen) and DH5$\alpha$ (pPProGreen) to 300 mM NaCl resulted in 9- and 47-fold induction, respectively, in $proU$-gfp expression (Table 1). The higher level of $proU$-gfp induction in DH5$\alpha$ (pPProGreen) than in DH5$\alpha$ (pVProGreen) provides additional
evidence for the existence of an upstream activating region. The enhanced responsiveness of DH5α (pPProGreen) relative to DH5α (pVProGreen) also indicates that the 612-bp proU promoter fragment is preferable to the 331-bp proU promoter fragment for use as a reporter of bacterial exposure to water deprivation.

Figure 1. Map of pPProGreen (15.2 kb). The promoter proU was directionally cloned from the proU expression vector pOSEX4 (19) into the broad-host-range pPROBE-KT vector (38). The rrnB transcriptional terminators, with one copy (T1) downstream and four copies (T4) upstream of the fusion, surrounded the PproU-gfp fusion. All of the restriction enzyme sites shown, including HindIII (H), SalI (S), BamHI (B), and EcoRI (E), were unique in pPProGreen.

Figure 2. Map of the proU promoter regions in plasmids A) pPProGreen and B) pVProGreen. The proU promoter contains an upstream activating region (UAR) and a silencer (S) located in the proV gene. The transcriptional (+1) and translational (+64) start sites are shown.

Table 1. The fluorescence of DH5α (pVProGreen) and DH5α (pPProGreen) cells that were grown on solid K medium with or without NaCl amendment.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>NaCl Concentration</th>
<th>Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α (pVProGreen)</td>
<td>0 mM</td>
<td>3.00±0.00</td>
</tr>
<tr>
<td></td>
<td>300 mM</td>
<td>28.33±1.45</td>
</tr>
<tr>
<td>DH5α (pPProGreen)</td>
<td>0 mM</td>
<td>5.33±0.88</td>
</tr>
<tr>
<td></td>
<td>300 mM</td>
<td>251.33±6.84</td>
</tr>
</tbody>
</table>

a Values were similar for cells grown in liquid K medium
b Values represent the mean fluorescence (rfu) ± standard error of the mean (SE) of 3 samples.
Two additional plasmids were constructed to confer constitutive \textit{gfp} expression in control strains. Plasmids containing \textit{gfp} driven by the promoter of the lactose operon (P\textsubscript{lac}) or the promoter of the neomycin phosphotransferase gene of Tn5 (P\textsubscript{nptII}) were constructed as described in the materials and methods; these plasmids were designated pVLacGreen and pPNptGreen, respectively. Expression of \textit{P\textsuperscript{nptII}-gfp} resulted in detectable fluorescence in all three strains: BRT98 (pPNptGreen) (143 rfu), DH5\textalpha{} (pPNptGreen) (65 rfu), and B728a (pPNptGreen) (34 rfu). In contrast, \textit{P\textsuperscript{lac}-gfp} resulted in detectable fluorescence in BRT98 (pVLacGreen) (524 rfu) and in DH5\textalpha{} (pVLacGreen) (144 rfu), but not in B728a (pVLacGreen). \textit{P\textsuperscript{lac}-gfp} expression appeared to be constitutive in both DH5\textalpha{} (pVLacGreen) and BRT98 (pVLacGreen) based on the absence of detectable induction by IPTG (data not shown). One possible reason for this may be because of the titration of the Lacl repressor due to the presence of multiple copies (6-8) of the plasmid (22).

\textbf{Osmoinduction of P\textsubscript{proU}-gfp in broth-grown \textit{E. coli} cells.}

We characterized the effect of NaCl concentration on \textit{P\textsubscript{proU}-gfp} expression in DH5\textalpha{} (pPProGreen) in broth-grown cells. Previous studies of \textit{P\textsubscript{proU}} expression in \textit{Salmonella typhimurium} examined induction by a single NaCl concentration (13, 19, 32) or by multiple concentrations (14, 19), with the latter demonstrating that \textit{P\textsubscript{proU}} expression varies with NaCl concentration. We observed a consistent increase in the \textit{P\textsubscript{proU}-gfp} expression as the NaCl concentration in the growth medium increased from 0 to 400 mM, with a 15-fold increase at 400 mM NaCl (Fig. 3A). The \textit{proU} promoter in DH5\textalpha{} (pPProGreen) was not induced by NaCl concentrations greater than 400mM (Fig. 3A), as was observed previously in \textit{S. typhimurium} (14). DH5\textalpha{} (pPProGreen) did not grow at salt concentrations greater than 500 mM NaCl.

A time course study using fluorometry demonstrated that induction of \textit{P\textsubscript{proU}-gfp} in DH5\textalpha{} (pPProGreen) occurred rapidly. In previous studies with \textit{E. coli} and \textit{S. typhimurium}, \textit{P\textsubscript{proU}} induction occurred within 10 min following exposure to NaCl (14, 33). We observed a detectable increase in \textit{P\textsubscript{proU}-gfp} expression in DH5\textalpha{} (pPProGreen) within 50 min following the addition of NaCl to broth-grown cells (data not shown); the relatively long period probably resulted from the time required for GFP folding (18).
Osmoinduction of \(P_{proU}gfp\) in broth-grown \(P. agglomerans\) and \(Ps. syringae\) cells.

BRT98 (pPProGreen) and DH5\(\alpha\) (pPProGreen) were similar in their basal level of \(P_{proU}gfp\) transcription in \(\frac{1}{2}\)-21C broth. At NaCl concentrations of 200 mM or greater, \(P_{proU}gfp\) was induced more in BRT98 (pPProGreen) than in DH5\(\alpha\) (pPProGreen) (Fig. 3A-B). Similar to DH5\(\alpha\) (pPProGreen), BRT98 (pPProGreen) exhibited a threshold NaCl concentration above which it did not exhibit further increases in fluorescence (Fig. 3B), as well as was inhibited in growth by NaCl concentrations greater than 500 mM.

B728a (pPProGreen) exhibited a significantly lower basal level of \(P_{proU}gfp\) transcription in \(\frac{1}{2}\)-21C broth (0 mM NaCl) than did either DH5\(\alpha\) (pPProGreen) or BRT98 (pPProGreen) (\(P<0.05\)). For example, in one study, the fluorescence of B728a (pPProGreen) in \(\frac{1}{2}\)-21C broth was 3.3±0.3 rfu, whereas that of DH5\(\alpha\) (pPProGreen) and BRT98 (pPProGreen) were 33.3±2.2 and 29.3±0.3 rfu, respectively. Although the maximum level of \(P_{proU}gfp\) induction in B728a (pPProGreen), which was achieved with 500 mM NaCl (Fig. 3C), was significantly less than in DH5\(\alpha\) (pPProGreen) or in BRT98 (pPProGreen), which were achieved with 400 and 300 mM NaCl, respectively (\(P<0.05\)), the induction ratio for \(P_{proU}gfp\) in B728a (pPProGreen) was larger than in the other strains at these NaCl concentrations (Fig. 3A-C). Similar to DH5\(\alpha\) (pPProGreen) and BRT98 (pPProGreen), B728a (pPProGreen) could not grow in \(\frac{1}{2}\)-21C broth with NaCl concentrations greater than 500mM.

The fluorescence of the strains DH5\(\alpha\) (pVLacGreen), BRT98 (pVLacGreen), and B728a (pPNptGreen) was not affected by the NaCl concentration in the medium (Fig. 3A-C). This indicates that the increased fluorescence observed in the \(P_{proU}gfp\) containing strains was due to \(P_{proU}gfp\) induction and not due to a direct effect of NaCl on the fluorescence of the GFP protein, or to an effect of NaCl on some aspect of the cell's physiology that influenced GFP fluorescence.

Osmoinduction of \(P_{proU}gfp\) in surface-grown cells.

We compared the effect of NaCl concentration on \(P_{proU}\) expression in planktonic (broth-grown) cells (Fig. 3A-C) versus surface-grown (plate-grown) cells (Fig 3D-F). The basal level of \(P_{proU}gfp\) expression in cells grown on \(\frac{1}{2}\)-21C plates was similar to that in cells grown in \(\frac{1}{2}\)-21C broth (data not shown). In DH5\(\alpha\) (pPProGreen) cells, \(P_{proU}\) was induced more by NaCl concentrations up to 400 mM in cells from plates than in cells from broth (Fig. 3A,D). Similarly, \(P_{proU}\) was induced more in BRT98 (pPProGreen) and in B728a (pPProGreen) in
cells from plates than in cells from broth at comparable NaCl concentrations. In contrast to the two enteric species, BRT98 (pPProGreen) and DH5α (pPProGreen), the strain B728a (pPProGreen) exhibited a dramatic increase in fluorescence as the NaCl was increased from 300 to 700 mM in the plates (Fig. 3D-F), and B728a (pPProGreen) grew on plates at NaCl concentrations greater than 500 mM.

To identify the lower limit of detection by the biosensors, we exposed BRT98 (pPProGreen), DH5α (pPProGreen), and B728a (pPProGreen) to NaCl concentrations between 0 and 100 mM. \( P_{proU}\text{-gfp} \) expression in all three species detectably increased over the range from 25 to 100 mM NaCl (Fig. 3D-F inserts). In an additional experiment, the minimum NaCl concentration that significantly increased cellular fluorescence in DH5α (pPProGreen) and B728a (pPProGreen) was 10 mM and in BRT98 (pPProGreen) was 15 mM \( (P<0.05) \) (data not shown).
Figure 3. Induction of P\textsubscript{pro}-gfp in cells that were grown in ½-21C broth (A, B and C) or on ½-21C plates (D, E and F) containing various concentrations of NaCl. A, D) E. coli DH5\textalpha{} (pPProGreen) (○), DH5\textalpha{} (pVLacGreen) (□); B, E) P. agglomerans BRT98 (pPProGreen) (○), BRT98 (pVLacGreen) (□); and C, F) Ps. syringae B728a (pPProGreen) (○), B728a (pPNptGreen) (□). The inserts show induction over a narrower range of NaCl concentrations. The fluorescence of the cells was measured with a fluorometer. The induction ratio was the mean fluorescence of the cells relative to the mean fluorescence of cells grown with 0 mM NaCl. Values represent the mean ± standard error of the mean (SE) (n=3).
**P_{proU}gfp induction by a diversity of solutes.**

In addition to NaCl, the solutes KCl and Na\textsubscript{2}SO\textsubscript{4} induced P\textsubscript{proU}gfp expression in all three bacterial species over a range of water potentials (data not shown). For example, DH5\textalpha{} (pPProGreen) and BRT98 (pPProGreen) cells were induced 2- to 3-fold by NaCl, KCl and Na\textsubscript{2}SO\textsubscript{4} levels conferring a water potential of -1 MPa. P\textsubscript{proU}gfp thus responded to high external osmolarity rather than to ion toxicity. The proU promoter similarly responded to high external osmolarity in *S. typhimurium* (14). The strains BRT98 (pPProGreen), DH5\textalpha{} (pPProGreen), and B728a (pPProGreen) were also grown on plates with the nonpermeating solute PEG 8000 (47), which limits water availability but does so by exerting pressure on the outer membrane rather than on the cytoplasmic membrane (16). PEG 8000 has been used as a dehydrating agent in many microbial and plant studies (e.g. 2, 10, 16), and is thought to better simulate conditions in low water content habitats than do permeating solutes. Although DH5\textalpha{} (pPProGreen) did not grow on 1/2-21C plates with PEG 8000, BRT98 (pPProGreen) and B728a (pPProGreen) exhibited increasing P\textsubscript{proU}gfp expression with increasing PEG 8000 concentrations (Fig. 4). In fact, the level of induction by PEG 8000 in BRT98 (pPProGreen) was similar to the level of induction by NaCl at comparable water potentials (Fig. 3 and 4); for example, BRT98 (pPProGreen) was induced 34- and 35-fold by -1.5 MPa conditions imposed by 335 mM NaCl and 33% PEG 8000, respectively (Fig. 3E and 4). In B728a (pPProGreen), P\textsubscript{proU}gfp was induced approximately 48- and 100-fold by -1.5 MPa conditions imposed by NaCl and PEG 8000, respectively (Fig. 3F and 4).

![Figure 4. Induction of P\textsubscript{proU}gfp in cells that were grown on 1/2-21C plates containing various levels of polyethylene glycol (PEG) 8000. *P. agglomerans* BRT98 (pPProGreen) (•) and *Ps. syringae* B728a (pPProGreen) (△) were grown for 24 h and the fluorescence intensity of cell suspensions was measured using a fluorometer. The induction ratio was the mean fluorescence of the cells relative to the mean fluorescence of cells grown with 0% PEG 8000. Values represent the mean ± SE (n=3).](image)
Spatial distribution of induced biosensor cells on bean leaf surfaces.

The spatial distribution of *P. agglomerans* and *Ps. syringae* cells exhibiting constitutive *gfp* expression was evaluated to identify the leaf surface regions that were colonized by these species under both high moisture and low moisture conditions. Although individual bacterial cells could often be visualized, the cells were also often in such close proximity to one another that the fluorescence appeared to be confluent. After 24 h of colonization under moist conditions, BRT98 (pVLacGreen) cells were visible as individual cells on the planar regions of plant cell surfaces. BRT98 (pVLacGreen) and B728a (pPNptGreen) cells were visible in epidermal cell junctions, particularly near veins, at the base of the hairlike trichomes and glandular trichomes, and in water droplets. Microcolonies of BRT98 (pVLacGreen) and B728a (pPNptGreen) were visible on both the abaxial and adaxial leaf surfaces. After 4 and 8 h of drying, i.e. at 4 and 8 h after transferring plants from a moisture tent to the laboratory bench, BRT98 (pVLacGreen) and B728a (pPNptGreen) cells were visible as confluent fluorescence in some epidermal cells junctions, in 'trails' between trichomes, and within circular regions that spanned multiple plant cells.

The spatial distribution of induced biosensor cells was evaluated based on the presence of visible fluorescence. After 24 h of colonization under moist conditions, neither BRT98 (pPProGreen) nor B728a (pPProGreen) exhibited detectable fluorescence (Fig. 5A). After 4 h of drying, the BRT98 (pPProGreen) and B728a (pPProGreen) cells in the epidermal cell junctions exhibited a gradient of increased fluorescence with increasing distance from the vein, and sometimes exhibited a gradient of fluorescence in circular regions spanning multiple plant cells, with the most intense fluorescence near the outer edge of the rings (Fig. 5B). Although after 4 h of drying all of the regions that were visibly colonized with BRT98 (pVLacGreen) and B728a (pPNptGreen) fostered induced cells of BRT98 (pPProGreen) and B728a (pPProGreen), the induced biosensor cells were present in a smaller proportion of these sites than were the cells that constitutively expressed *gfp*, and fewer microcolonies were visible for B728a (pPProGreen) than for BRT98 (pPProGreen) on the leaf surface. In contrast to BRT98 (pVLacGreen) and BRT98 (pPProGreen), B728a (pPNptGreen) and induced B728a (pPProGreen) cells were uniquely visible in lines radiating out from the glandular trichomes.
Figure 5. Spatial distribution of cells exhibiting fluorescence due to P_{proU-gfp} induction on bean leaves (Phaseolus vulgaris). A) *P. agglomerans* BRT98 (pPProGreen) on a bean leaf after 24 h of incubation under moist conditions (no visible bacterial fluorescence), B) *P. agglomerans* BRT98 (pPProGreen) on a bean leaf 4 h after transferring the plants from moist conditions to ambient conditions (i.e. after 4 h of drying), C) *Ps. syringae* B728a (pPProGreen) on a bean leaf after 4 h of drying, D) *P. agglomerans* BRT98 (pPProGreen) on a bean leaf after 8 h of drying, and E) *Ps. syringae* B728a (pPProGreen) on a bean leaf after 8 h of drying. Leaves were examined by epifluorescence microscopy. The bacterial cells exhibited green or bluish-green fluorescence (A-E), the plant epidermal cells exhibited red autofluorescence (A-E), the leaf trichomes exhibited bluish-green (B-D) or blue (E) autofluorescence, and the stomata exhibited blue autofluorescence (E). (Magnification = 100X)

**Induction of P_{proU-gfp} in BRT98 (pPProGreen) on leaves.**

BRT98 (pPProGreen) was used to investigate the extent to which water is available to bacteria on leaf surfaces under conditions that favored evaporation of water from the leaf surface. The mean fluorescence of the BRT98 (pPProGreen) populations recovered from each of several leaves immediately following inoculation (0 min of drying) was used as a baseline measurement to determine the extent of induction at each subsequent sampling time. After the indicated drying time, the plants were enclosed in pre-moistened tents to prevent further drying and to allow for GFP maturation to a fluorescent state (50). Among the leaves sampled for each time point in experiment 1, there was relatively little leaf-to-leaf variation in the mean fluorescence of the recovered populations (Fig. 6A). The mean fluorescence of the cells recovered from each leaf consistently increased over the entire 30-
min period, although visible water loss from the leaves did not occur until between 10 and 15 min after inoculation. In fact, the increase in the mean fluorescence was significant after only a 5-min drying period (P < 0.05), indicating that a macroscopic evaluation of the presence of water is a poor predictor of water availability at the microscopic level. By 30 min, the mean fluorescence of the population had increased 1.47-fold relative to at 0 min (Fig. 6A). We generated a curve of the level of \( P_{proU-gfp} \) induction in plate-grown BRT98 (pPPProGreen) cells at NaCl concentrations of 0 to 300 mM using flow cytometry to measure fluorescence (data not shown). Based on this curve (\( y = 0.943e^{0.0089x} \), in which \( x = \) NaCl concentration and \( y = \) ratio of the fluorescence at a given NaCl concentration to that at 0 mM), at 30 min after inoculation, the average cell in the population recovered from leaves was exposed to a water potential that was equivalent to that imposed by approximately 55 mM NaCl. The variance of the fluorescence increased as the mean fluorescence increased, and the rate of this increase was similar to that observed with plate-grown cells. The mean forward scatter of the populations consistently decreased over the 30-min period examined, and a regression of the mean forward scatter over time showed that this decrease was significant (slope = -0.1, \( P = 0.01 \)). Forward scatter reflects cell size (24), thus this supports previous observations that bacterial cells decrease in size following their arrival on a leaf surface (J.-M. Monier and S.E. Lindow, Abstr. 7th International Symposium on the Microbiology of Aerial Plant Surfaces, abstr. 28, 2000) (C. Riffaud and C.E. Morris, Abstr. 7th International Symposium on the Microbiology of Aerial Plant Surfaces, abstr. 35, 2000). This size change, however, was not related to their increased fluorescence, based on the absence of a significant correlation between mean fluorescence and mean forward scatter.

In a replicate experiment, experiment 2, the monitoring period was extended to 4 h, and the control strain BRT98 (pVLacGreen) was included. Similar to experiment 1, there was relatively little leaf-to-leaf variation at any given time point, at least within the first hour (Fig. 6B). The mean fluorescence of the cells recovered from each leaf consistently increased over the first 35 min. Subsequent incubation under ambient conditions did not result in significant increases in the mean fluorescence of the populations. Similar to experiment 1, the mean fluorescence of the population significantly increased immediately following introduction of the bacterial cells to the leaf surface (\( P < 0.05 \)). In contrast to BRT98 (pPPProGreen), BRT98 (pVLacGreen) did not exhibit a significant increase or decrease in fluorescence over the 4-h period examined. For example, at any given time point, the ratio of the mean fluorescence for BRT98 (pVLacGreen) to that at 0 min was generally in the
range of 0.9 to 1.1. The mean fluorescence of the BRT98 (pPProGreen) cells recovered at 35 and 60 min had increased by 1.61- and 1.67-fold relative to at 0 min (Fig. 6B), which corresponded to an exposure of the average cell in the populations to water potentials equivalent to those imposed by NaCl concentrations of 67 and 71 mM, respectively, at these times. The mean forward scatter of both the BRT98 (pPProGreen) and BRT98 (pVLacGreen) populations decreased significantly over time (for both strains: slope = -0.02, P < 0.01), suggesting that the cells decreased in size (data not shown). Similar to in experiment 1, this size change was not related to the increased fluorescence based on the absence of a significant correlation between the mean fluorescence and the mean forward scatter (P < 0.05).

![Figure 6](image)

Figure 6. Induction of \( P_{\text{proU}} \cdot gfp \) in \( P. \text{agglomerans} \) BRT98 (pPProGreen) cells recovered from bean leaves at various times following inoculation. A) Experiment 1 in which the cells were monitored for 30 min (n=3), B) Experiment 2 in which the cells were monitored for 240 min (n=2). The induction ratio was the mean fluorescence of the cells relative to the mean fluorescence of the cells at 0 min. Values represent the mean ± SE for n samples. Data points indicated by the same letter do not differ by Fisher's Least Significant Difference test (P<0.05).

Subsequent plant studies were done in an environmental chamber so we were able to control the humidity conditions. Experiments were performed at 45%, 70% and 95% RH. Similar levels of induction were seen for BRT98(pPProGreen) populations at all three levels.
of humidity; however, the amount of time required for the same level of induction to be reached lengthened at the higher humidity levels (see Chapter 3).

Discussion

We have developed GFP-based biosensors that can be used to quantify bacterial water deprivation. These biosensors allow us to measure the water availability actually sensed by bacteria in a given habitat, rather than simply the general water status of the habitat. Some key features of these biosensors include the following. They respond in a quantitative manner to water deprivation. They are sufficiently sensitive to detect water deprivation levels as low as those imposed by only 10 to 15 mM NaCl. Their detection range is moderately broad, with the upper limit approaching the water deprivation levels that inhibit growth for at least one bacterial species. They contain the $P_{proU}gfp$ fusion on a multi-copy, broad-host-range plasmid, and this effectively amplifies the visible response to water deprivation and facilitates its introduction into a variety of bacterial species. The high level of stability of this plasmid in the species tested, both in the absence and presence of high osmolarity, ensures its maintenance under conditions without antibiotic selection (see Appendix 1). And lastly, the absence of a requirement for specific regulatory elements for regulation of the $proU$ promoter suggests that $P_{proU}$ is likely to respond to water deprivation in many bacterial species; this is supported by our finding that $P_{proU}gfp$ responded to water deprivation in *E. coli*, *P. agglomerans*, and *Ps. syringae*, as well as in *Xanthomonas campestris pv. vesicatoria* (unpublished data).

Our results with two distinct $proU$ promoter constructs confirmed that an approximately 200-bp upstream activating region (UAR) is required for high level induction of the $proU$ promoter. The presence of the entire UAR region resulted in approximately 10-fold higher fluorescence levels than the presence of only half the region when the cells were grown with 300 mM NaCl. Our results also provide evidence supporting Lucht and Bremer’s (32) finding that a silencer, which represses basal expression in low osmoticum media is in the first 200 bp following the transcriptional start site.

A key assumption with these biosensors is that cellular fluorescence reflects the activity of the $proU$ promoter. This is supported by the fact that exposure of the biosensor cells to NaCl concentrations from 0 to 300 mM correlated with both an increase in the amount of GFP protein present, as detected by Western blots (data not shown), and an increase in cellular fluorescence. More importantly, for the control cells, which were the same strains
as the biosensors but which exhibited constitutive gfp expression, exposure to NaCl concentrations from 0 to 500 mM did not cause detectable changes in cellular fluorescence. This indicates that the cellular fluorescence of the strains was not significantly influenced by water deprivation-induced changes in cell physiology. Such changes include a decreased rate of growth (26), which was observed with B728a at high NaCl concentrations, but was not associated with a change in the fluorescence of B728a (pPNptGreen).

This is the first report that the proU promoter from E. coli functions in species other than E. coli and S. typhimurium. The basal level of Ppro expression in Ps. syringae was lower than in E. coli, but was similar in P. agglomerans and E. coli. This could be due to the effect of poor promoter recognition or low transcriptional efficiency of an E. coli promoter in Ps. syringae. The osmoresponsiveness of the proU promoter in Ps. syringae provides support for the hypothesis that proU osmoregulation does not require specific regulatory proteins, although it is possible that Ps. syringae provided such proteins.

For all three strains tested, plate-grown cells exhibited higher levels of induction than broth-grown (i.e. planktonic) cells at comparable NaCl concentrations. Surface-grown cells are known to differ physiologically from planktonic cells (43). In fact, this difference has been demonstrated to influence bacterial survival following introduction onto plant leaves (52). In this study, the similarity in the level of PproU-gfp expression in broth- and plate-grown cells when NaCl was absent indicated that surface-associated and planktonic cells differed not in their basal proU expression, but rather in the extent of proU induction. In most terrestrial habitats, bacterial cells are present on surfaces and thus physiologically, they are likely to be more similar to surface-grown than planktonic cells.

As a model system for evaluating the effectiveness of the bacterial biosensors, we examined bacterial exposure to water deprivation during the period of evaporation that followed bacterial introduction onto aerial leaf surfaces. We reasoned that during this period, the visible water film on a leaf disappears, and thus many of the bacteria on the leaf should become limited for water. We focused on water availability specifically following an inoculation event because we could control the presence of water on a leaf during this period, and because it seemed highly probable that at least some of the inoculum cells would arrive in sites that would not support the continued presence of water.

The microscopy studies demonstrated that bacteria were exposed to water deprivation on bean leaves under the conditions tested, and they illustrated the spatial distribution of these cells. Only a subset of the colonized sites contained biosensor cells that were
exposed to sufficient levels of water deprivation to cause detectable fluorescence. We observed a gradient of decreasing water availability with increasing distance from a vein, demonstrating that the proximity of cells to a vein, where water likely collects, influenced their access to water. The circular regions of fluorescence on the leaves suggest that as the water droplets on a leaf surface evaporated, the bacteria in the receding edge of those droplets became limited for water.

We used flow cytometry to quantify bacterial exposure to water deprivation on leaves; in these studies we examined the fluorescence of each cell in the populations recovered from the leaves. We used BRT98 (pPProGreen) because it exhibited a sufficiently high basal level of fluorescence to allow uninduced cells to be distinguished from the indigenous bacteria and particulates that were also recovered from the leaf surface. *E. coli* DH5α was of less interest because it is not a normal inhabitant of leaf surfaces, and *Ps. syringae* B728a (pPProGreen) exhibited a basal level of fluorescence that was too low to effectively separate uninduced cells from the indigenous bacteria. Future studies with this strain, and other strains that similarly exhibit a low basal level of *proU* expression, will involve introduction of a second fluorescence marker. The consistent level of fluorescence of the control strain BRT98 (pVLacGreen) on leaves indicated that the bacteria did not undergo physiological changes that affected their fluorescence, nor were they exposed to environmental conditions that detrimentally affect GFP fluorescence. Such conditions include very low pH (4 to 5.5) and high pH (>12) (7), strong reducing agents (21), and hydrogen peroxide concentrations greater than 1% (21). The significant increase in the fluorescence of the BRT98 (pPProGreen) cells in the 30 to 60 min period following inoculation indicated that many cells were limited for water during this period, as was predicted. The relatively low leaf-to-leaf variability suggests uniformity in the macroenvironment of the leaves, which may have resulted, in part, from a very low leaf density in the pots. The low variability further suggests that biologically-based differences in leaf-water relations were relatively small among the leaves.

We predicted that the variability in water availability among microsites on a leaf should be reflected in the variability in fluorescence. We assumed that the variance in the fluorescence values of a population of cells from a leaf is composed of two components, the variance inherent to cells following induction to a given level of water deprivation and the variance that results from the heterogeneity among the microsites in water availability. We estimated the former by examining the variance of cells in a homogenous habitat, i.e. in a
laboratory culture. We compared the rate at which the variance increased as the mean fluorescence of the population increased and found that both the cultured cells and the cells from leaves exhibited the same rate of increase over a range of mean fluorescence values. The absence of a detectable increase in the variance in fluorescence of cells from leaves over that of cells in culture indicates that under the conditions tested, the heterogeneity in water availability among the microsites on a leaf, which was detected by microscopy, was not detectable in the flow cytometry analysis of the cells removed from leaves. The two methods of examining the biosensor cells thus each provided unique information. The simplest explanation for a relatively uniform rate of increase in fluorescence across a population is that the decreased water availability following inoculation resulted primarily from the dissolution of solutes that were on the leaf surface preceding inoculation, and the subsequent decreases in water availability resulted from an increase in the concentration of solutes following evaporation.

The level of induction of the average cell in the population indicated that the cells were exposed to a water potential equivalent to that imposed by only 50-60 mM NaCl in the 30-min period following arrival on the leaf surface. This is a fairly low level of water deprivation, i.e. it is not sufficiently high as to cause cell death or perhaps even reduce growth. If we assume that bacteria and solutes are uniformly distributed throughout a water layer on a leaf, and we assume that solutes remain on a leaf when the water evaporates, then an increase in water deprivation equivalent to that imposed by an increase from 19.2 to 55 mM NaCl, which occurred in this study between 5 and 30 min following inoculation, would correspond to a 65% decrease in the water volume on the leaf in that 25 min period. We have estimated that bean leaves with an area of 50 cm² hold about 0.5 g of water. If that water was evenly spread across the surface of the leaf, then a 65% decrease in water volume would correspond to a decrease in the water film thickness from 100 μm to 35 μm.

Macroscale measurements of the water potential of plant leaves are highly variable, but are generally much lower than the levels sensed by the biosensor on the leaves in this study. On average, the bacteria on the leaves in this study were exposed to water potentials in the range of -0.05 to -0.42 MPa. In contrast, in published studies, leaf psychrometer measurements of leaf water potential ranged from -0.2 to -2.07 MPa, depending on the species, time of day, and location of the leaf in the canopy ((9), (25), (36), (23), (11)). The fact that the bacteria in this study were generally exposed to less stressful levels of water deprivation than would be predicted by these leaf psychrometer
measurements suggests that the bacteria localized to microsites on leaves that were particularly well hydrated.

In summary, these studies demonstrated that a $P_{pou}gfp$ fusion-based biosensor was effective at making microscale measurements of water availability to bacteria on bean leaves. The measurements indicated that under the environmental conditions selected, bacteria on the leaves were exposed, on average, to water potentials as low as -0.42 MPa. This exposure level was unlikely to limit bacterial growth or survival on leaves, based on bacterial growth under water deprivation conditions on plates. Potential uses of these biosensors include evaluating the role of water deprivation in bacterial population dynamics on plants and in other natural habitats, and identifying the effect of exopolysaccharides and biofilm formation on bacterial exposure to water deprivation.

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References


Chapter 3. Availability of Water to Bacteria on Bean Leaves

introduction

Aerial leaf surfaces support multiple species of yeast, fungi and bacteria. The most abundant of the organisms are bacteria, many of which are beneficial to the plant. The microorganisms found on the leaf surface are exposed to daily fluctuations in environmental factors such as temperature, water availability, UV radiation and available nutrients. The rapid changes in these factors on the aerial leaf surface can be very harsh and may affect the growth and survival of the microorganisms that are present. However, many bacteria tolerate the fluctuating and often extreme environment and develop populations of significant size (e.g., \(10^7\) CFU/g leaf weight). The surviving cells may be located in niches where they are protected from the ambient environment; this is supported by the complex 3-dimensional surface of leaves. The leaf is also surrounded by a boundary layer in which conditions may differ from the ambient environmental conditions. Currently we have a poor understanding of the actual conditions that microbes encounter on leaf surfaces and their impact on the survival of bacterial cells.

We have investigated the level of water deprivation encountered on bean (\(\textit{Phaseolus vulgaris}\) L.) leaves by cells of the epiphytic bacterial species \textit{Pantoea agglomerans} strain BRT98 under controlled conditions. We sampled during a 4 h period after inoculation when visible evaporation occurred. Using a previously constructed fusion composed of the water-responsive promoter \textit{proU} and the reporter gene \textit{gfp} (2), which encodes a green fluorescent protein (GFP), we were able to quantify the water potential encountered by BRT98 on bean leaves under laboratory and field conditions. Although BRT98 cells encountered very little water deprivation on leaves in the lab, we found suggestive evidence that they were strongly deprived in the field.

Materials and Methods

Bacterial strain, plasmids and growth conditions

The bacterial strain used for this study was \textit{P. agglomerans} strain BRT98 (4). The strain was grown on Luria-Bertani (LB) medium (14) for routine maintenance at 28°C, or on one of two low-osmoticum media, ½-21C medium (8, 18) or K medium (10) for inocula preparation. The strain was resistant to rifampin, and the plasmids that were used (described below) conferred resistance to kanamycin. Rifampin and kanamycin were added
at a concentration of 50 μg/ml, while cycloheximide was added at a concentration of 100 μg/ml to eliminate the growth of fungi on plates.

The construction of the plasmids pPProGreen and pVLacGreen was described previously (2). The pVNptGreen plasmid was constructed by PCR amplification of the gfp gene from the pGreenTIR plasmid (15) using the primers 5'-CGAGCTCGAATTCT and 5'-GAGCTCGAATTCC-3. The 752-bp EcoRI amplified fragment was cloned into pVSP61 (9, 11, 21) with a HindIII-PvuII fragment containing the lacZ promoter deleted. A 364-bp SalI fragment with the nptII promoter for Tn5 (5, 12) was cloned upstream of the gfp gene.

**Plant material and growth conditions**

*Phaseolus vulgaris* L. cv. Bush Blue Lake 274 bean plants (Burpee, Warminster, PA) were used for both chamber and field experiments. For chamber experiments, 7 seeds per pot were planted in a 1:2:1 peat-perlite-soil potting mixture and grown at 45%, 70% or 95% relative humidity (RH) and 28°C with a 12-h photoperiod of 350 μeinsteins m⁻² sec⁻¹. For the field experiment, 48 seeds were planted in each of 8 blocks (8 by 36 in) on 7/25/01. The experimental area was surrounded by 3 ft of an unplanted zone and blocks were separated by 3 ft. The field experiment was conducted at the Horticultural Research Farm at Iowa State University near Gilbert, IA on 8/8/01. To release gfp-marked recombinant strains, we obtained a permit from the U.S. Department of Agriculture Animal and Plant Health Inspection Service.

A Spectrum Technologies, Inc. WatchDog data logger (Model 150) (Plainfield, IL) was used to record temperature and RH in the chamber. A Campbell Scientific (Logan, UT) CD10 data logger was used to monitor relative humidity, temperature, leaf wetness, wind speed and rainfall in the field, as described previously (17).

**Plant inoculation and sampling**

The inocula for plant studies were prepared by growing the strains on ½-21C, resuspended to a concentration of 10⁸ cells/ml in ½-21C broth and bean plants with fully expanded primary leaves were inoculated by submerging, as previously described (2). Immediately following inoculation of plants from the growth chamber, plants were returned to the chamber. After various drying times (0, 10, 20, 30, 60, 90, 120 and 180 min), a pot was removed and the plants were covered with a pre-moistened plastic tent for 2 h to allow time
for proper GFP folding (20). Three leaves per time point were sampled and placed separately in test tubes containing 20 ml of ½-21C broth.

For the field experiment, plants were inoculated by spraying both the abaxial and adaxial leaf surfaces. To prevent environmental contamination, plants were inoculated under an enclosed chamber constructed of polyethylene sheets and PVC tubes. At various times (0, 15, 30, 60, 120, 180 and 240 min), 12 leaves were collected and each leaf was incubated dry in a test tube for 2 h and was then submerged in 20 ml of ½-21C broth.

**Characterization of \( P_{\text{proU-gfp}} \) expression in planta**

The characterization of \( P_{\text{proU-gfp}} \) expression has been previously described (2). Briefly, leaf samples were sonicated for 7 min to recover bacteria. Bacteria for each sample were enumerated by removing a 50 \( \mu \)l aliquot of the leaf sonicate and plating onto LB agar. The remaining sonicate was concentrated by filtering through a 0.22 \( \mu \)m membrane filter and resuspending in 3 ml of ½-21C. Samples were analyzed by flow cytometry.

Expression of \( \text{proU-gfp} \) in bacterial aggregates developed on leaves was compared to expression in solitary cells on leaves. The samples were taken at 1, 2 and 5 days after inoculation in the laboratory experiments, and at 1 and 2 days after inoculation in the field experiments. The isolation of aggregates was performed as previously described with slight modifications (16). Specifically, 5 leaves were collected per replicate and added to a flask containing 100 ml of ½-21C broth. The samples were agitated at 25 rpm for 3 min to suspend the aggregates and solitary cells in the broth. These cell suspensions were filtered first through a 5 \( \mu \)m membrane filter to collect aggregates and then through a 0.2 \( \mu \)m filter to collect cells and cell clusters < 5 \( \mu \)m. The cells were recovered from the filters by agitation in 3 ml of ½-21C broth. Cells were analyzed by flow cytometry as previously described (2).

**Growth dynamics of BRT98 in culture**

Plate-grown cultures of BRT98 were exposed to both NaCl and PEG 8000 to assess the effect of water deprivation on the cells. Cells were exposed to between 0 and 2100 mM NaCl and PEG 8000 on ½-21C plates. Cells were grown on ½-21C plates at 28°C for 24 h. For studies on solid medium, cells were diluted to \( 10^4 \) cells/ml on ½-21C plates amended with either NaCl or PEG 8000. Plates were examined every 3 h to determine the initial appearance of colonies. Growth rates and colony diameters were also measured for all concentrations.
Results and Discussion

To investigate the extent to which epiphytic bacteria are exposed to water deprivation on bean leaves, the fluorescence of BRT98(pPProGreen) was monitored under conditions that favored various extents of evaporation. We previously reported that the mean fluorescence of cells recovered from leaves increased over a 30 min drying period following inoculation, with the increase being significant after only 5 min of drying (2). This previous study was conducted on the laboratory bench where environmental conditions could not be controlled. In the current study, plants were placed in a controlled environmental chamber and were subjected to 45%, 70% and 95% RH (Fig. 1A). The fluorescence of cells recovered from leaves following 0 min of drying differed among the studies. To account for this, the mean fluorescence of cells recovered at 0 min of drying was used to normalize the fluorescence at subsequent time points within an experiment. At 45% RH, the mean fluorescence of the recovered cells increased significantly by 20 min in one study (Fig. 1A) and by 60 min in a replicate study (data not shown). In contrast, at 95% RH, the mean fluorescence of the recovered cells did not increase significantly until 120 min or later (Fig. 1A). Using the previously established relationship between the level of \( P_{proL}^{cr} gfp \) induction in plate-grown cells and NaCl concentration (0 to 300 mM) in the plates (\( y = 0.943e^{0.0086x} \), in which \( x = \) NaCl concentration and \( y = \) ratio of the fluorescence at a given NaCl concentration to that at 0 mM) (2), we were able to quantify the amount of water deprivation encountered by these BRT98(pPProGreen) cells on bean leaves. Based on the 1.78- to 2.14-fold increase in the mean fluorescence of cells exposed to 45% RH at 90 to 180 min relative to at 0 min, the BRT98(pPProGreen) cells encountered water deprivation levels of 64 (-0.29 MPa) to 88 (-0.40 MPa) mM NaCl equivalence. The mean fluorescence relative to 0 min increased 1.97-fold (equivalent to 73 mM NaCl (-0.33 MPa)) by 180 min for cells exposed to 70% RH, and 2.06-fold (equivalent to 79 mM NaCl (-0.35 MPa)) by 180 min and 2.59-fold (equivalent to 103 mM NaCl (-0.46 MPa)) by 240 min for cells exposed to 95% RH. The BRT98(pPProGreen) population sizes on the leaves did not significantly change during the period studied under any of the selected RH levels (Fig. 1B).

BRT98(pPProGreen) eventually encountered similar levels of water deprivation when present on leaves exposed to 45%, 70% and 95% RH; however, the rate at which the water availability decreased differed among treatments. At 45% RH, cells reached the fluorescence maximum more rapidly than they did at 70 and 95% RH, indicating that 45% RH supports a more rapid rate of drying than higher RH levels, as would be expected.
fact that the water potentials that were sensed by the populations were similar under all 3 RH conditions suggests that the leaf has a certain level of hydration regardless of the changes in the ambient air conditions within the range of conditions employed in this study.

The strain BRT98(pVNptGreen) carrying a constitutively expressed nptII-gfp fusion was used as a control strain to evaluate the effect of environmental conditions on the fluorescence of the GFP protein. After applying BRT98(pVNptGreen) cells to bean leaves and incubating the leaves under 45, 70 and 95% RH conditions, the mean fluorescence of the BRT98(pVNptGreen) cells increased between 1.1- and 1.2-fold over the 180 min period under all of the RH levels examined (data not shown). The limited change in the control strain fluorescence suggests that exposure to environmental factors in the chamber did not affect GFP fluorescence.

We observed a difference in fluorescence between the cells in the inoculum suspension and the cells recovered from leaf samples after 0 min of leaf exposure to drying. The ratio of the fluorescence of the recovered cells to the inoculated cells decreased with increasing RH (Fig. 2). The ratio of the fluorescence of cells from the inoculum and the 0 min sample was significantly correlated with the RH (%) \(y = -0.005x + 1.254, R^2 = 0.23, P < 0.003,\) in which \(x = \) relative humidity (%) and \(y = \) ratio of the fluorescence). Since we calculated the increase in fluorescence at each drying time after normalizing to the fluorescence of the cells at 0 min, we may actually be underestimating the level of water deprivation encountered by the bacteria on bean leaves.
Figure 1. Fluorescence of P$_{proU}$-gfp in *P. agglomerans* BRT98 (pPProGreen) cells and population sizes recovered from bean leaves at various times following inoculation. Two experiments were conducted at 45% RH (●) and the other experiment not shown, one experiment at 70% RH (■), and two experiments at 95% RH (▲ and △). A) Induction of P$_{proU}$-gfp in cells. The induction ratio was the mean fluorescence of the cells at a given NaCl concentration relative to the mean fluorescence of the cells at 0 min. Data points marked with an asterisk differ significantly from the value at 0 h within each experiment. B) Mean population sizes of cells recovered from bean leaves. The values represent the mean ± SE for *n* = 3 samples.
Figure 2. Influence of the RH conditions under which the plants were grown and incubated on the ratio between the fluorescence of the inoculum cells and the fluorescence of cells recovered after 0 min of drying and 2 h of incubation under moist conditions.

Biofilms, which are aggregates of microorganisms attached to each other and/or a surface, are common in many environments including on leaf surfaces (7, 16). Biofilms may protect microorganisms from environmental stresses, such as water stress, suggesting that an organism in a biofilm would be exposed to less water deprivation than solitary cells. To test this hypothesis, we investigated the amount of water deprivation encountered by BRT98(pPProGreen) in aggregates, collected on 5 µm filters, versus as single cells, collected by passing the filtrate from the 5 µm filter onto a 0.2 µm filter, both in an environmental chamber and in the field. Samples were viewed microscopically to establish that aggregates and single cells were collected. The cells recovered on the filters were vortexed to release single cells before analysis by flow cytometry. During the field study, BRT98(pPProGreen) fluorescence was significantly higher for solitary cells than for cells recovered from aggregates 24 h after inoculation onto leaves (Table 1). Although this difference was not significant for cells recovered at 48 h, a similar trend was observed (Table 1). The fluorescence of single cells increased significantly between 24 and 48 h, whereas the fluorescence of cells in aggregates did not. During analysis of field samples,
high numbers of large aggregates consisting of both fluorescing and non-fluorescing cells were visualized. Based on the observation that uninduced BRT98(pPProGreen) cells exhibit visible fluorescence, we assumed that the non-fluorescing cells were indigenous bacteria. It is likely that the bacteria introduced onto the leaf surface joined pre-existing aggregates based on that BRT98 formation of aggregates on bean leaves requires longer than 48 h, at least under controlled conditions (17). Members of a bacterial community found in an aggregate may be afforded protection against limited water availability by the matrix in which they are embedded. Among the cells recovered from leaves in the environmental chamber, we observed no significant differences between the fluorescence of the cells released from aggregates and the fluorescence of cells recovered as solitary cells. The probable absence of pre-existing aggregates on leaves under laboratory conditions likely precluded the detection of cells in aggregates at 24 h and 48 h post-inoculation. Supporting this, we detected very few aggregates among cells recovered from leaves under laboratory conditions, and the aggregates that were present consisted of only a few fluorescent cells and no non-fluorescent cells. Although no differences were observed between the fluorescence of single cells and those cells found in aggregates in the environmental chamber, future studies should be done to investigate the differences at a later time when aggregates are confirmed to be present.

Table 1. Fluorescence of bacterial cells recovered as solitary cells or from aggregates from inoculated leaves under field conditions. Values represent the mean ± SE for n = 3 samples. Values indicated by the same lower case letter within a column, whereas those indicated by the same upper case letter between columns do not differ by Fisher's LSD test (P < 0.05).

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Filter type</th>
<th>Sampling time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>Solitary cells</td>
<td>0.2 μm</td>
<td>34.00 ± 2.55 aA</td>
</tr>
<tr>
<td>Cells from aggregates</td>
<td>5.0 μm</td>
<td>21.75 ± 1.44 bA</td>
</tr>
</tbody>
</table>

We investigated the water deprivation encountered by BRT98(pPProGreen) on bean plants under field conditions. One experiment was initiated during the morning hours when dew was still visible, the temperature was 21 to 23°C, and the RH was 90 to 96%. By 15 min after inoculation, the mean fluorescence of the cells recovered from the leaves had increased significantly to 1.07-fold relative to 0 min (equivalent to 12 mM NaCl (-0.05 MPa)) (Fig. 3A). A second experiment was initiated in the afternoon when the temperature was 30 to 31°C and the RH was 65 to 70%. By 15 min, the mean fluorescence of the cells
significantly increased to 1.44-fold (equivalent to 42 mM NaCl (-0.19 MPa)) (Fig. 3A). The bacteria encountered more water deprivation during the afternoon experiment than in the morning experiment, thus validating the assumption that epiphytic bacteria encounter more water deprivation during hot and dry periods than during cool and moist periods.

Figure 3. Fluorescence of *P. pmu*-gfp in *P. agglomerans* BRT98 (pPProGreen) cells and population sizes recovered from bean leaves in field conditions at various times following inoculation in a morning experiment (●) and in an afternoon experiment (■). A) Induction of BRT98(pPProGreen) cells, and B) mean epiphytic population sizes of BRT98(pPProGreen) The values represent the mean ± SE for *n* = 3 samples.
In both experiments, the mean fluorescence of the cells eventually decreased, with the decrease occurring within the first 30 min of the afternoon experiment, but only after an hour in the morning experiment. The fluorescence eventually decreased to lower than the initial fluorescence readings (0 min) in both experiments. These results suggest that the environmental conditions in the field may be detrimental to GFP stability or fluorescence. These conditions may include sunlight and relatively high temperatures. The observed reductions in fluorescence could also be due to the presence of proteases induced in cells following inoculation onto leaves. Another possibility for this loss of fluorescence in the field could be the loss of the GFP protein from the cell. Previous researchers have reported the loss of cytosolic proteins less than 100 kDa from the cell during times of stress, including osmotic and matric stress (1, 22). The proteins are thought to be lost through mechanosensitive channels that are formed rapidly after exposure to these stresses (23). Of these possibilities for the observed loss of GFP fluorescence, the exposure to sunlight may be the most likely. Subsequent studies demonstrated that GFP fluorescence decreased in the presence of sunlight (Chen and Beattie, unpublished data).

To investigate the population dynamics over time in the field, we determined colony forming units by plating onto LA agar and viable counts by staining with propidium iodide. In the afternoon experiment, propidium iodide (PI) was added to an aliquot of the samples at 0, 60, 120 and 240 min to differentiate nonviable from viable cells. Propidium iodide easily diffuses into cells that are lacking membrane integrity and binds irreversibly to DNA. The samples were analyzed using flow cytometry and fluorescent beads for counting, and GFP and PI fluorescence in cells were easily separated due to the differences in emission. We found that by 60 min we were underestimating the number of viable cells by using plate counts, although the general population dynamics were similar when estimated by plate counts and viable cell count by flow cytometry (Fig. 4).

To evaluate the physiological response of BRT98 to low water potentials, we grew cells both in liquid amended with NaCl and on solid medium amended with NaCl or PEG 8000. PEG 8000 has been used as a dehydrating agent in many applications (3, 6, 8, 19). We examined multiple growth parameters including the time before colonies became visible on plates, the growth rate, growth inhibition, maximum colony diameter and the number of culturable cells.
Growth of BRT98 on a solid medium was investigated to determine the physiological response of BRT98 to low water potentials under conditions that mimicked growth on the plant surface. Although there was a significant increase in fluorescence of BRT98(pPProGreen) cells during the drying period on leaves, water deprivation levels equivalent to those encountered by BRT98(pPProGreen) on leaves did not kill the organism in culture. However, the growth rate and lag time of BRT98 in culture were altered by this level of water deprivation. As the water potential decreased, the growth characteristics changed similarly for both BRT98 and BRT98(pPProGreen). When BRT98 cultures were grown on plates in the absence of exogenous NaCl, the length of time until colonies became visible, i.e., the lag time, was 18.5 h. The lag time increased with increasing NaCl concentrations (Fig. 5). A similar relationship was observed when cells were grown with PEG 8000 on plates (data not shown). Growth rates were not significantly different for cells grown on plates with 0 or 200 mM NaCl, but were when grown on plates with 0 or 200 mM NaCl.
PEG 8000 (Table 2). These results indicate that matric stress imposed by PEG 8000 had a greater impact on growth rate than a comparable osmotic stress, as has been observed previously with *E. coli* (13). No significant differences were observed in colony diameter for cells grown on ½-21C agar amended with NaCl concentrations in the range of 0 to 600 mM NaCl (data not shown). In contrast, the addition of PEG 8000 at concentrations of 25, 100 and 200 mM significantly increased colony diameter compared to colonies on plates without PEG 8000 (data not shown). One possible explanation for the increase in diameter with an increase in PEG 8000 when combined with the negative impact of PEG 8000 on growth rate is that EPS is produced in response to elevated matric stress. When cultures of BRT98(pPProGreen) were plated onto ½-21C medium amended with NaCl, the percentage of culturable cells decreased at NaCl concentrations of 400 mM or greater (Table 3). The number of culturable cells at a given NaCl concentration was calculated by comparing the number of colonies at a given water potential to the number of cells that were culturable at 0 mM NaCl (Table 3). The addition of 25 to 300 mM NaCl actually increased the number of colonies compared to 0 mM, although only the increase at 300 mM was statistically significant (Table 3). Colonies were not detected on ½-21C solid medium at NaCl concentrations of 600 mM or greater (based on Table 3).

Table 2. Growth rates (mm/h) for BRT98(pPProGreen) on ½-21C solid medium amended with NaCl or PEG 8000. Values indicated by the same letter within each column do not differ by Fisher’s LSD test (P < 0.05).

<table>
<thead>
<tr>
<th>NaCl Concentration (mM)</th>
<th>PEG 8000 Concentration (%)</th>
<th>Water potential (MPa)</th>
<th>Growth rates on ½-21C solid medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>With NaCl</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.044 ± 0.003 a</td>
</tr>
<tr>
<td>25</td>
<td>5</td>
<td>-0.11</td>
<td>0.071 ± 0.002 a</td>
</tr>
<tr>
<td>50</td>
<td>10</td>
<td>-0.22</td>
<td>0.035 ± 0.003 a</td>
</tr>
<tr>
<td>100</td>
<td>15</td>
<td>-0.45</td>
<td>0.037 ± 0.002 a</td>
</tr>
<tr>
<td>200</td>
<td>26</td>
<td>-0.90</td>
<td>0.035 ± 0.003 a</td>
</tr>
</tbody>
</table>

^aNT Not tested
Figure 5. The length of time before visible colonies were observed for BRT98(pPProGreen) on ½-21C plates amended with NaCl. The relationship between lag time and NaCl concentration for cells grown on ½-21C agar was $y = 17.118e^{0.0018x}$ $R^2 = 0.97$, where $y = $ lag time (h) and $x = $ NaCl concentration (mM).

Table 3. Effect of water potential on the culturability of BRT98(pPProGreen) cells. Values indicated by the same letter within each column do not differ by Fisher's LSD test ($P < 0.05$).

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>Water potential (MPa)</th>
<th>Percent of colonies that developed relative to the number on media with 0 mM NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>100 ± 3 b</td>
</tr>
<tr>
<td>25</td>
<td>-0.11</td>
<td>125 ± 7 ab</td>
</tr>
<tr>
<td>50</td>
<td>-0.22</td>
<td>116 ± 3 ab</td>
</tr>
<tr>
<td>100</td>
<td>-0.45</td>
<td>136 ± 9 ab</td>
</tr>
<tr>
<td>150</td>
<td>-0.67</td>
<td>NT$^a$</td>
</tr>
<tr>
<td>200</td>
<td>-0.90</td>
<td>110 ± 4 ab</td>
</tr>
<tr>
<td>300</td>
<td>-1.35</td>
<td>144 ± 23 ab</td>
</tr>
<tr>
<td>400</td>
<td>-1.79</td>
<td>21 ± 4 c</td>
</tr>
<tr>
<td>450</td>
<td>-2.02</td>
<td>NT$^a$</td>
</tr>
<tr>
<td>500</td>
<td>-2.24</td>
<td>7 ± 1 d</td>
</tr>
</tbody>
</table>

$^a$NT Not tested

In experiments with plants in an environmental chamber, BRT98(pPProGreen) cells encountered water potentials as low as approximately -0.45 MPa (100 mM NaCl equivalence) on the aerial leaf surface. The growth experiments in culture suggested that this level of water deprivation does significantly reduce the growth rate of cells if present as osmotic stress but does not if present as matric stress. This level of water deprivation also
increases the lag time for cells by about 2 h. Although these growth dynamics are altered slightly, this level of water deprivation did not hinder the survival based on culturability of BRT98 in the tests in culture. Unfortunately, we were only able to quantify the level of water deprivation encountered in the field within the first hour before the GFP fluorescence was lost from the cells. However, comparing the culturability data from the field experiment to a standard curve generated from the culturability data of BRT98(pPProGreen) on ½-21C agar, cells sampled at 240 min from the field experiment were exposed to approximately 450 to 520 mM NaCl equivalence. This level of water deprivation would significantly hinder growth of BRT98(pPProGreen).

Acknowledgements

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References


Chapter 4. Water Relations During the Hypersensitive Response

Introduction

Plants are able to sense and respond to environmental stresses such as invasion by microorganisms. One plant defense mechanism involves recognition between a specific plant resistance ($R$) gene product and the corresponding pathogen avirulence ($avr$) gene product. This mechanism of plant defense, called the hypersensitive response (HR), involves a rapid induction of localized host cell death. In a pathogen-plant interaction in which either an $avr$ gene or its corresponding $R$ gene is absent, recognition does not occur. In the absence of recognition and under favorable environmental conditions, a pathogen can colonize a plant and possibly induce disease.

Bacterial-induced defenses in plants include membrane and plant cell structure changes that can eventually lead to localized plant cell death. Reactive oxygen species (ROS) including hydrogen peroxide and superoxide anions accumulate rapidly during both HR and pathogenesis, although to a greater extent during HR. Hydrogen peroxide has been shown to accumulate in cell walls adjacent to bacteria (5, 23), and to be distributed heterogeneously throughout the sites in the intercellular spaces during HR. These ROS promote membrane damage via lipid peroxidation. Simultaneously, potassium ions are rapidly transported out of the plant cell and hydrogen ions are transported in, causing acidification of the cell and eventual depolarization of the membrane. During the HR, cell membranes lose structural integrity, as evidenced by extensive electrolyte leakage. During pathogenesis, electrolytes are also released, but to a lesser extent.

Although the initial plant defense responses are similar during an incompatible response (HR) and a compatible response (disease), pathogen growth is restricted during HR but is not when the plant-pathogen interaction is compatible. During pathogenesis, bacterial populations often attain concentrations as high as $10^7$ to $10^8$ CFU/cm$^2$, whereas in HR, populations plateau at 10- to 100-fold lower levels (19, 41). Based on previous studies with *Pseudomonas syringae* in bean leaves or *Arabidopsis thaliana* leaves, bacterial populations during HR were restricted from further growth after 24 h at high inoculum levels (11, 19, 41) as would be predicted if they were exposed to high concentrations of ROS. Interestingly, bacterial populations generally did not decrease subsequent to this growth cessation (11, 19, 41). The mechanisms driving these differences in population size during these two plant responses are not known.
Many have hypothesized that pathogens induce changes in the intercellular environment in susceptible plants, such as by inducing nutrient leakage (2, 18, 32). For example, increased electrolyte leakage by *Erwinia amylovora* was observed in apple leaves (9). This initial leakage of electrolytes is thought to lead to subsequent leakage of sucrose which could act as a nutrient supply to the pathogen. Pathogens may also suppress plant defense responses. Pathogens can secrete elicitors that induce defenses in the plant.

Several researchers have demonstrated the ability of bacterial effector proteins to suppress plant responses. Effectors are a group of elicitors that are secreted through the type III secretion apparatus (8). For example, the effector molecules AvrPphC, AvrPphF and VirPphA in *Pseudomonas syringae pv. phaseolicola* (24, 38), and the effectors AvrPtoB, HopPtoD2 and AvrPto in *Ps. syringae pv. tomato* strain DC3000 (1, 14, 44), were able to suppress host defense mechanisms.

Pathogen growth is probably actively restricted during the HR. This restriction could result from phytoalexins, hydrogen peroxide, or, as will be explored in this work, from restrictive physical conditions in the apoplast. Any or all of these phenomena may occur concurrently. Several researchers have observed that in an incompatible reaction the plant cells nearest the bacteria collapse, turn brown and produce a yellow-green fluorescence (15, 28). This yellow-green fluorescence is due to the presence of certain phytoalexins that are diffusible and accumulate in the leaves when pathogens are present (16, 17). Similarly, hydrogen peroxide and superoxide anions are produced in response to invading pathogens. Researchers have reported that hydrogen peroxide accumulates to varying degrees at distinct sites in leaves (5, 23). Based on the bacterial population dynamics previously reported, bacterial populations during HR seem to be restricted from further growth after 24 h at high inoculation levels (11). Although population decreases as large as 50% occurred within 8 h after inoculation (5), decreases in the subsequent hours and days have not been reported.

Very little is known about the availability of water to bacteria during the progression of HR or the development of disease. We have measured the water potential that bacteria sense in the presence and absence of an interacting *avr-R* gene pair. Here we report that *Pseudomonas syringae pv. tomato* strain DC3000 experiences water potentials during HR that are equivalent to water potentials that severely restrict DC3000 growth in culture. In contrast, in a compatible interaction, DC3000 experiences water potentials that are equivalent to those that enhance DC3000 growth in culture.
Materials and Methods

Bacterial strains, plasmids and media

The bacterial strains and plasmids used in this study are listed in Table 1. Strains were grown in Luria-Bertani (LB) medium (29) for routine maintenance and for plate counts. A low osmoticum medium, K medium (25), amended with either NaCl or H₂O₂ was used for induction assays. Antibiotics and concentrations (µg/ml) used are as follows: rifampin (50), kanamycin (50), streptomycin (20), spectinomycin (20), chloromphenicol (30), tetracycline (20), and ampicillin (50). To eliminate growth of fungi cycloheximide (100 µg/ml) was used in plates. To construct pPProlce, a 612-bp EcoRI-BamHI fragment containing the inducible proU promoter was cloned from the pOSEX4 vector into pPROBE-KI upstream of the inaZ gene. The plasmid pPNptlce was constructed by PGR amplification of the constitutive nptll promoter from Tn5. The 365-bp SalI fragment containing the nptll promoter was inserted upstream of the inaZ gene in the vector pPROBE-KI. The plasmids were mobilized into DC3000, DC3000 hrcc (previously named hrpH) and A506 by triparental matings with the helper plasmid pRK2073.

Plant growth and inoculation

Arabidopsis thaliana plants were grown on LC1 Sunshine Mix (Sun Gro Horticulture, Pine Bluff, AR) soil in an 11-h light/13-h dark cycle at 25°C and 20-25 % RH. Twelve seeds were planted in 3.5 in square pots using a 00 artist brush. All assays were performed with 5 to 6 week old plants of A. thaliana Columbia (Col-0) (genotype, RPS2IRPS2 RPM1IRPM1) (Lehle Seeds, Round Rock, TX) except where indicated differently. An RPS2 mutant (genotype rps2irps2 RPM1IRPM1) was provided by Dr. Andrew Bent (University of Wisconsin-Madison) and was previously described (4, 26). Strains were grown on K medium plates for 2 days at 24°C, resuspended in 10mM MgSO₄ to an optical density at 600nm (OD₆₀₀) of 0.1 and diluted 10-fold before inoculation. Cells were introduced into the abaxial side of leaves using a 1-ml blunt syringe until a 1 cm² region was visibly infiltrated (12).

Measurement of ice nucleation activity

Samples to be evaluated for ice nucleation activity (INA) were diluted in K broth. INA was tested at -9°C by a droplet freezing technique (27, 39). Specifically, three 10-µl droplets of each of several 10-fold dilutions were placed on a paraffin-coated aluminum foil
boat in a circulating ethylene glycol (50%) bath, and the dilution that yielded 1 to 2 frozen droplets after 2 minutes was identified. Forty 10-μl droplets of this dilution were used to estimate both the number of ice nuclei/ml in the sample, as previously described (39), and the INA (ice nuclei/bacterium) after the bacteria in the sample were enumerated on LB agar with the appropriate antibiotic. For two in planta experiments, a 90% ethanol bath that resulted in higher INA values was used instead of the 50% ethylene glycol bath. The emulsion beneath the foil that occurs in an ethylene glycol bath did not allow for cooling to the desired temperature.

Measurement of \( P_{proU}\)-inaZ activity in culture

The effect of NaCl concentration on \( P_{proU}\)-inaZ expression was assessed by using cells grown on solid media containing 0 to 500 mM NaCl (0 to -2.4 MPa) (21). Cultures were resuspended in K broth to a density of \( 10^3 \) cells/ml and were plated onto K plates. Cultures were grown at 24°C until colonies were approximately 2 mm in diameter; this required 72-120 h depending on the NaCl concentration.

The effect of \( H_2O_2 \) concentration on \( P_{proU}\)-inaZ expression was assessed by using mid-log phase cultures in K broth containing 0, 0.3, 0.6 and 0.9 mM \( H_2O_2 \). Cultures were grown in the absence of \( H_2O_2 \) at 24°C until dense and were sub-cultured and grown for an additional 5 h. The samples were adjusted to an \( OD_{600} \) of 0.125, diluted 10-fold and then exposed to distinct concentrations of \( H_2O_2 \) for 2 h. Ice nucleation activity was assessed as described above.

Measurement of \( P_{proU}\)-inaZ activity in planta

After inoculation, plants were placed in a growth chamber under conditions of 25°C, 11-h light/13-h dark cycle and 20% relative humidity (RH) for 48 hours. For each sample, approximately 1 g of leaf material was removed, weighed and homogenized to completion with 5 ml of K broth for 40 seconds using a Kleco Ball Mill (Visalia, CA). The INA and bacterial population size in each sample were determined as described above.
Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/Plasmid</th>
<th>Relevant Characteristics</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>Nl'</td>
<td>Gibco-BRL,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rockville, MD</td>
</tr>
<tr>
<td><em>Pseudomonas syringae pv. tomato</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DC3000</td>
<td>Rf'</td>
<td>(31)</td>
</tr>
<tr>
<td>DC3000 hrcC</td>
<td>hrcC::Tn5Cm, Rf', Cm'</td>
<td>(45)</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
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<td></td>
</tr>
<tr>
<td>A506</td>
<td>Rf'</td>
<td>(42)</td>
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<tr>
<td>Plasmid</td>
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<td></td>
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<tr>
<td>pPROBE-KI</td>
<td>Broad-host-range plasmid, Km', MCS upstream of promoterless inaZ gene</td>
<td>(30)</td>
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<td>pPROBE-KI'</td>
<td>pPROBE-KI with the MCS in the reverse orientation</td>
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<tr>
<td>pOSEX4</td>
<td>contains the <em>Escherichia coli</em> proU promoter, Ap'</td>
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<td>pPROBE-KT containing the Tn5 nptll promoter fused to gfp</td>
<td>(3)</td>
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<td>pPProlce</td>
<td>pPROBE-KI containing the proU promoter from pOSEX4 fused to inaZ</td>
<td>This study</td>
</tr>
<tr>
<td>pPNptlce</td>
<td>pPROBE-KI the nptll promoter from Tn5 fused to inaZ</td>
<td>This study</td>
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<td>pRK2073</td>
<td>Tra', Mob', Sm', Sp'</td>
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<td>pLAFR3 containing a 1.4-kb fragment with avrRpt2, Tc'</td>
<td>(41)</td>
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<td>(12)</td>
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<td>pLAFR3 containing a 2.2-kb PstI fragment with avrB, Tc'</td>
<td>(37)</td>
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</tbody>
</table>

Ap', ampicillin resistance; Cm', chloramphenicol resistance; Km', kanamycin resistance; Nl', nalidixic acid resistance; Rf', rifampin resistance; Sm', streptomycin resistance; Sp', spectinomycin resistance; Tc', tetracycline resistance

Characterization of DC3000 growth in culture

The effect of NaCl concentration on the growth of DC3000 and various derivatives was assessed by using cells plate-grown cultures exposed to 0 to 500 mM NaCl (0 to -2.4 MPa) and from broth cultures exposed to 0 to 1000 mM NaCl (0 to -4.6 MPa). Cells from broth cultures were grown in K broth at 28°C for 24 h, and were adjusted to a density of 10⁸ cells/ml using standard curves relating cell number to OD₆₀₀. After diluting to 10⁸ cells/ml in K broth containing various NaCl concentrations, samples were taken at 0.5, 1, 2, 4, 8, 12,
17, 20, 24, 28, 40 and 48 h and were plated onto LB agar containing antibiotics. The length of the lag phase and growth rate in K broth was estimated. Cells were also grown on solid K medium (K agar) for 48 h at 28°C, and a suspension of 10⁷ cells/ml in K broth was plated onto K agar containing various NaCl concentrations. Time until the initial appearance of colonies, designated the lag time, was determined for each NaCl concentration by examining plates every three hours after inoculation. Colony diameters (mm) were measured at least twice a day to determine the growth rate at each NaCl concentration. The number of colonies on plates without NaCl was used as a reference to estimate the number of culturable cells at each NaCl concentration.

**Results**

**DC3000 is exposed to significantly lower water potentials during an HR reaction than during pathogenesis.** To evaluate the water potential sensed by bacteria in incompatible versus compatible interactions with plants, a plasmid containing a proU-inaz fusion was introduced into DC3000 and DC3000 derivatives containing various cloned avirulence genes. The log (INA) of these strains was directly related to the water potential to which the bacteria were exposed (Fig. 1a and 1b). We previously reported that a proU-gfp fusion was induced by distinct permeating solutes, including Na₂SO₄, KCl, and the non-permeating solute PEG8000, demonstrating that P₉₆₉ responds to low water potentials rather than specifically to NaCl (3). Forty-eight hours after bacterial cells were infiltrated into *A. thaliana* (Col-O) leaves, DC3000 exhibited a significantly higher INA when it contained the *avrRpf2*-encoding plasmid pLH12 (Log (INA) = -1.57 ± 0.13), than when it did not (Log (INA) = -4.84 ± 0.28). This was observed in two additional replicate experiments. The relations between ice nucleation activity and water potential on solid media (Fig. 1) were used to estimate the water potentials sensed by the bacteria in planta based on the Log (INA) values measured in planta. For example, in the experiment described above, DC3000 cells were exposed to a water potential of −0.14 ± 0.10 MPa whereas DC3000(pLH12) cells were exposed to −1.58 ± 0.05 MPa. A similar difference between the strains was observed in 7 experiments involving *A. thaliana* (Col-O) (Fig. 2A, experiments A-G). The disruption of *avrRpt2* in DC3000(pLH12Δ) resulted in the bacteria sensing water potentials similar to those sensed by DC3000 on Col-O (Fig. 2A, experiments D-F). Similarly, when DC3000(pLH12) was introduced into an *A. thaliana RPS2* mutant, the cells sensed water
potentials similar to those sensed by DC3000 on Col-O or the RPS2 mutant (Fig. 2A, experiments H-J).

On Col-O, DC3000 consistently sensed lower water potentials when it expressed any of the 3 avirulence genes avrRpt2, avrRpm1 and avrB than when it did not (Fig. 2A, experiments D-G). In 3 of 4 experiments, DC3000 (pPProlce/pK48-8) was exposed to significantly lower water potentials than either DC3000(pPProlce/pLH12) or DC3000(pPProlce/pPGS002), suggesting a greater impact of avrRpm1 than of avrRpt2 or avrB on bacterial access to water.

A control plasmid, pPNptlce, which conferred constitutive inaZ expression, was used to determine if exposure to limited water availability affected the activity of the InaZ protein. The increase in Log (INA) for proU-inaZ activity in an HR when compared to in the absence of an HR ranged from 645- to 1,902-fold while the increase in npt-inaZ activity ranged from 2- to 3-fold (Table 2). Based on these results, exposure to low water potential does not alter activity of the ice protein.

Table 2. Log (INA) values for three independent A. thaliana studies. Experiments B and E are as described in the legend to Fig. 2. Experiment 3 is similar to experiments C – J in Fig. 2. Values represent the mean ± standard error of the mean for n = 3 samples. For pPProlce-containing strains within an experiment, Log(INA) values followed by the same letter do not differ significantly by Fisher’s LSD test (P < 0.05). The same is true for the pPNptlce-containing strains within an experiment.

<table>
<thead>
<tr>
<th>Strain Identification</th>
<th>Experiment B</th>
<th>Experiment E</th>
<th>Experiment 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC3000(pPProlce)</td>
<td>-3.97 ± 0.18 a</td>
<td>-3.99 ± 0.26 a</td>
<td>NT</td>
</tr>
<tr>
<td>DC3000(pPProlce/pLH12Ω)</td>
<td>NT</td>
<td>-3.72 ± 0.25 a</td>
<td>NT</td>
</tr>
<tr>
<td>DC3000(pPProlce/pLH12)</td>
<td>-1.16 ± 0.54 b</td>
<td>-1.05 ± 0.09 b</td>
<td>NT</td>
</tr>
<tr>
<td>DC3000(pPNptlce)</td>
<td>-2.21 ± 0.77 a</td>
<td>NT</td>
<td>-2.38 ± 0.09 a</td>
</tr>
<tr>
<td>DC3000(pPNptlce/pLH12Ω)</td>
<td>NT</td>
<td>NT</td>
<td>-2.08 ± 0.06 b</td>
</tr>
<tr>
<td>DC3000(pPNptlce/pLH12)</td>
<td>-1.95 ± 0.68 a</td>
<td>NT</td>
<td>-2.53 ± 0.05 a</td>
</tr>
</tbody>
</table>

NT = Not tested
Figure 1. Relationship between water potential (-MPa) and Log (INA) values on solid K plates for A) DC3000(pPProlice) and B) DC3000(pPProlice/pLH12). Regression analysis yielded the following equations: \( y = -2.85x - 5.23 \) (\( R^2 = 0.79 \)) for DC3000(pPProlice) and \( y = -2.40x - 5.35 \) (\( R^2 = 0.94 \)) for DC3000(pPProlice/pLH12).
The decrease in water availability to bacteria occurred rapidly during an HR. The water potential sensed by bacteria in an HR decreased significantly in the 4 hours following infiltration, with further decreases thereafter (Fig. 3C). In the first 4 h following inoculation, DC3000(pPPro1ce) experienced a significant decrease of 0.2 MPa, whereas DC3000(pPPro1ce/pLH12) experienced a 0.45 MPa decrease. By 72 h, DC3000(pPPro1ce) cells were exposed to water potential of $-1.02 \pm 0.08$ MPa, whereas DC3000(pLH12) cells were exposed to a water potential of $-1.89 \pm 0.04$ MPa. By 4 h for the control strain, DC3000(pPPro1ce) and DC3000(pPPro1ce/pLH12), the Log(INA) values increased by 1.4-fold and 1.5-fold, respectively (Fig. 3D), as compared to 3.5- and 12.1-fold for DC3000(pPPro1ce) and DC3000(pPPro1ce/pLH12), respectively.

Non-pathogens encountered higher water potentials in planta than did DC3000. To measure the water potential sensed by a nonpathogens within a leaf, we infiltrated A. thaliana Col-O plants with Ps. fluorescens strain A506. Like DC3000, A506 does not exhibit natural ice nucleation activity. In 3 out of the 4 studies, A506(pPPro1ce) encountered significantly higher water potentials than did DC3000(pPPro1ce) (Fig. 4A). Typically, DC3000(pPPro1ce) was exposed to 1.3- to 1.7-fold lower water potential than was A506(pPPro1ce).

A non-pathogenic mutant of DC3000 was also examined. This hrcC mutant, which was defective in pathogenicity and HR induction on A. thaliana, also encountered significantly higher water potentials than did DC3000(pPPro1ce) (Fig. 4A). The hrcC mutant encountered similar water potentials as A506 (Fig. 4).
Figure 3. Dynamics of the $P_{proU-inaz}$ (A-C) and $P_{npt-inaz}$ (D and E) in DC3000 in the presence or absence of the avirulence gene $avrRpt2$ over 3 days study period. (A) Ice nucleation activity, (B) Population sizes and (C) Water potential for DC3000(pPProlce) and DC3000(pPProlce/pLH12). (D) Ice nucleation activity and (E) Population sizes for DC3000(pPNptlce) and DC3000(pPNptlce/pLH12). Values represent the mean ± standard error of the mean for $n = 3$ samples.
Figure 4. *P. putida* *inaZ* induction and population sizes of A506(pPPro1ce), DC3000 *hrcC* (pPPro1ce) and DC3000(pPPro1ce) 48 h after infiltration. (A) Water potential (MPa) sensed by cells based on the standard curves: $y = 0.04x - 5.34$ ($R^2 = 0.87$) for A506 and $y = 0.03x - 3.76$ ($R^2 = 0.89$) for DC3000 *hrcC* where $x =$ Water potential (MPa) and $y =$ Log (INA). (B) Population sizes. Values represent the mean ± standard error of the mean for $n = 3$ samples. Data points indicated by the same letter do not differ within each experiment by Fisher's LSD test ($P < 0.05$).
Hydrogen peroxide did not affect *proU* expression or ice nucleation activity. Following a 2 h exposure to H$_2$O$_2$, DC3000(pPProlce) exhibited a small induction (2-fold) by 0.6 mM H$_2$O$_2$ in one study, but no significant induction even with the addition of 0.9 mM H$_2$O$_2$ in a second study (Table 3). The trend in the second study was actually a slight decrease in *proU-inaN* expression with increasing H$_2$O$_2$ concentrations. These results suggest that hydrogen peroxide was not an inducing signal for P$_{proU}$. To test the impact of hydrogen peroxide on the activity of the reporter protein InaZ, we exposed DC3000(pPNptlce) to H$_2$O$_2$. The Log(INA) values for DC3000(pPNptlce) were similar at all H$_2$O$_2$ concentrations, indicating that hydrogen peroxide does not impact the activity of InaZ.

Table 3. The impact of hydrogen peroxide on *proU-inaN* expression and ice nucleation activity in two independent experiments. Data points indicated by the same letter do not differ within each experiment by Fisher's LSD test (P < 0.05).

<table>
<thead>
<tr>
<th>Strain</th>
<th>H$_2$O$_2$ Concentration (mM)</th>
<th>Log(INA) Experiment 1</th>
<th>Log(INA) Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC3000(pPProlce)</td>
<td>0.0</td>
<td>$-4.10 \pm 0.02$ a</td>
<td>$-4.73 \pm 0.27$ a</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>$-4.26 \pm 0.13$ a</td>
<td>$-4.93 \pm 0.15$ a</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>$-3.79 \pm 0.04$ b</td>
<td>$-5.64 \pm 0.60$ a</td>
</tr>
<tr>
<td></td>
<td>0.9</td>
<td>NT</td>
<td>$-5.31 \pm 0.24$ a</td>
</tr>
<tr>
<td>DC3000(pPNptlce)</td>
<td>0.0</td>
<td>$-4.88 \pm 0.34$ a</td>
<td>$-3.07 \pm 0.42$ a</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>$-5.29 \pm 0.09$ a</td>
<td>$-2.64 \pm 0.24$ a</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>$-4.96 \pm 0.32$ a</td>
<td>$-2.99 \pm 0.22$ a</td>
</tr>
<tr>
<td></td>
<td>0.9</td>
<td>NT</td>
<td>$-3.10 \pm 0.17$ a</td>
</tr>
</tbody>
</table>

NT = Not tested

Population Sizes. Bacterial population sizes were significantly smaller during HR than during pathogenesis (Fig. 2B), as been observed in many previous studies. For example, in experiment E (Fig. 2B), DC3000(pPProlce/pLH12) populations were only 2.7 % the size of the DC3000(pPProlce) populations. In the same study, populations of DC3000(pPProlce/pK48-8) and DC3000(pPProlce/pPGS002) were 13.1 % and 9.7 % the size of the DC3000(pPProlce) population, respectively. In general, the population sizes of the DC3000 variants during an incompatible interaction at 48 h after inoculation were 3 to 13 % the size of those during a compatible interaction. Similar differences between incompatible and compatible interactions were observed with DC3000 variants containing pPProlce and pPNptlce (Fig. 2B, 3B and 3E). Populations of DC3000 were considerably greater than populations of A506 and DC3000 *hrcC* mutant (Fig. 4). DC3000(pPProlce)
established populations that were 167-fold greater than those of A506(pPProlce) and 665-fold greater than those of DC3000 \textit{hrcC}(pPProlce).

In 5 independent experiments (experiments B, D, E, F and G in Fig. 2B), there was a significant linear correlation between the Log(CFU/g leaf) values and the water potential ($P < 0.01$). Although the large population sizes in the plants involved in compatible interactions contributed to this correlation, the correlation was still observed even when these population were removed from the analyses. On average, a decrease in water potential of $-0.9$ MPa correlated with a 10-fold decrease in CFU/g leaf (Fig. 5).

![Graph showing the relationship between bacterial population size and water potential.](image)

Figure 5. Relationship between bacterial population size and the water potential encountered by that population in \textit{A. thaliana} Col-O leaves ($y = -0.99x + 6.63$, $R^2 = 0.89$). Data are representative of the results in 4 other independent studies.
Physiological response of DC3000 to low water potentials. We evaluated the effects of various water potentials on several aspects of bacterial growth including the lag time, i.e., the time before colonies became visible on plates or before the turbidity began increasing in broth, the growth rate, growth inhibition, culturability following short-term and long-term exposure, maximum colony diameter, and cell size. In these experiments the water potential was altered using NaCl amendment.

In general, the strains DC3000 and DC3000(pPProlce) grown on solid media responded similarly to decreasing water potentials in all of the growth characteristics examined. The lag time on K agar increased linearly from 31 h at 0 MPa to 42 h at -0.47 MPa and 76 h at -1.83 MPa (y = -25.07x + 30.51, where y = lag time (h) and x = water potential (MPa), R² = 0.94). The growth rate on K agar generally decreased as the water potentials decreased (Fig. 6). Slower growth rates occurred at -0.25 and -0.5 MPa, but the decrease was not significant until -0.5 to -0.9 MPa. Colony size was also affected by the decrease in water potential. When cells were grown on K agar with water potentials between 0 and -0.92 MPa, they established colonies that were > 1.8 mm in diameter (range: 1.86 to 3.31 mm). On K agar with water potentials of -1.38 and -2.29 MPa, the colony diameters were 1.38 and 0.37 mm, respectively, indicating that colony diameter decreased with decreasing water potentials. No detectable growth occurred on solid media with water potentials less than -2.3 MPa.

DC3000 and DC3000(pPProlce) were generally similar in their response to decreased water potentials when grown in liquid media as well. A lag phase was not detectable in K broth with water potentials of 0 and -0.92 MPa, but was at lower water potentials of -1.83 MPa and lower (Fig. 7). The growth rate (Log(CFU/ml)/h) at 0 MPa was 0.067 ± 0.002 but increased significantly to 0.085 ± 0.003 at -0.92 MPa. No significant growth or death occurred at water potentials of -1.83 and -2.74 MPa over a 48-h period, suggesting that these levels are sufficient to stop cell division but are not lethal. Similarly, no significant growth or death occurred at water potentials of -3.65 and -4.56 MPa over a 24 h period; however, after 24 h, the cells rapidly died or became unculturable (Fig. 7).
Figure 6. Growth rates (mm/hour) of DC3000 and DC3000(pPPro1ce) on K agar containing NaCl to confer various water potentials. Values indicated by the same letter within each experiment do not differ by Fisher's LSD test ($P < 0.05$).
Figure 7. Growth dynamics of DC3000 in K broth containing NaCl to confer various water potentials. These results are representative of those in 2 additional replicate experiments.

Exposure of a cell population to low water potential can differentially influence the cells in the population. We evaluated this by examining the proportion of a population that is able to multiply and form visible colonies, i.e. the proportion that was culturable. When a culture of DC3000(pPro1cI) was plated on K agar containing NaCl, the percent of culturable cells decreased as the water potential decreased (Table 4). Replicate experiments yielded similar results.
Table 4. Percentage of culturable DC3000(pPProlce) cells when exposed to distinct water potentials on K agar containing NaCl. Values indicated by the same letter within each experiment do not differ by Fisher's LSD test (P < 0.05).

<table>
<thead>
<tr>
<th>Water potential (MPa)</th>
<th>Percent comparison to colony number at 0 MPa (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DC3000</td>
</tr>
<tr>
<td>0.00</td>
<td>100.0 ± 0.0 a</td>
</tr>
<tr>
<td>-0.13</td>
<td>107.6 ± 10.4 a</td>
</tr>
<tr>
<td>-0.24</td>
<td>113.6 ± 14.4 a</td>
</tr>
<tr>
<td>-0.47</td>
<td>79.6 ± 24.6 a</td>
</tr>
<tr>
<td>-0.92</td>
<td>93.2 ± 8.7 a</td>
</tr>
<tr>
<td>-1.38</td>
<td>82.2 ± 16.9 a</td>
</tr>
<tr>
<td>-1.83</td>
<td>4.7 ± 0.7 b</td>
</tr>
<tr>
<td>-2.29</td>
<td>3.2 ± 0.3 b</td>
</tr>
</tbody>
</table>

Discussion

DC3000 encountered lower water potentials in an incompatible more than with a compatible plant-pathogen interaction. Using an ice nucleation assay, we were able to measure the expression level of proU-inaz in DC3000(pPProlce) after infiltration of A. thaliana leaves and use this expression level to estimate the water potential sensed by the bacteria in planta. We examined incompatible interactions using DC3000 containing the avr genes avrRpt2, avrRpm1 and avrB, which interact with the A. thaliana genes RPS2, RPM1 and RPM1, respectively. We examined three compatible interactions. First, A. thaliana Col-O and DC3000(pPProlce), which itself does not contain an avr gene that interacts with the R genes in A. thaliana Col-O. Secondly, A. thaliana Col-O and DC3000(pPProlce/pLH12Ω), which contains a disrupted avrRpt2 gene; and finally an A. thaliana RPS2 mutant and DC3000(pPProlce/pLH12), which encodes avrRpt2. In all cases, the results were consistent: cells of the Ps. syringae pv. tomato pathogen encountered significantly more water deprivation with incompatible interactions than with compatible interactions.

The saprophyte Ps. fluorescens A506 encountered water potentials of −0.3 to −0.4 MPa after infiltration into A. thaliana. In contrast, the pathogen DC3000 encountered significantly lower water potentials, −0.6 to −1.0 MPa, after infiltration into the compatible host Col-O. This suggests that the pathogen and/or the host actively modifies the intercellular environment during a compatible interaction. Based on growth characteristics in culture, DC3000 exhibited optimal growth at a water potential of −0.9 MPa, indicating that the altered water potential in the intercellular spaces may favor optimal DC3000 growth. In liquid medium, the growth rate and percent culturability of cells at −0.9 MPa were significantly higher than those of cells exposed to 0 MPa. During an HR, DC3000
encountered water potentials of $-1.3$ to $-2.3$ MPa. These levels of water deprivation were severe enough in culture to halt further growth of the cells.

The decreased water availability we observed during an HR could result from at least two distinct mechanisms: cells could experience high solute concentrations due to the release of ions, or cells could experience desiccation due to loss of water from the infection site. We propose two models to explain how the water availability decreases at the infection site during an HR (Fig. 8). In the first, the plant actively restricts xylem flow preventing nutrients and water from reaching the infected site; such host restriction of xylem flow to an infected site has been shown (43). If the stomates remain open during an HR, as has been demonstrated by some researchers (7, 20, 34), and transpiration continues, as has been shown (7), then the infected site will become desiccated. Such desiccation is thought to lead to visible cell collapse and wilting at the infection site (43). In the second model, the plant actively restricts the phloem flow while the xylem remains open (35). The xylem will, therefore, continue to deliver $K^+$ ions and water to the region. If the stomates remain closed in the infected area, as has been documented by some researchers (10, 13), transpiration will not occur and the $K^+$ and water will accumulate in the intercellular spaces. This accumulation will be amplified by the known loss of $K^+$ ions from the plant cells during HR. The associated loss of an osmotic gradient across the cytoplasmic membrane (2, 33) and resulting loss of water to the intercellular spaces could cause the loss of plant cell turgor, thus causing collapsing and wilting of the plant tissue. The increase of $K^+$ ions in the intercellular spaces would result in bacterial exposure to elevated osmotic stress. Our results demonstrate only that the water potential at the infected site is low, and can not distinguish between these two models.

In a compatible interaction, the water potential decrease was small but significant. This decrease likely was the result of the $K^+$ efflux that occurs during pathogenesis. This apoplastic increase in $K^+$ precedes the growth of the bacteria (2). The $K^+$ efflux is associated with an $H^+$ influx, and the resulting change in $H^+$ gradient could hinder the uptake of sucrose (2), resulting in an increase in sucrose availability in the intercellular spaces that could enhance bacterial growth (2). However, this mechanism for an increase in sucrose availability has been questioned on the basis that mesophyll cells may preferentially export, rather than import, sucrose into the intercellular spaces (40). More recent hypotheses describing the mechanism of increased nutrient availability in the apoplast focus on an
active role for bacterial effectors, which are transported into the plant cell cytoplasm, in the induction of nutrient release by plant cells.

**Model 1**

- **Stomates open**
- Outcome - Wilting due to loss of water from infected site
- Increase in transpiration

**Model 2**

- **Stomates closed**
- Outcome - Wilting due to accumulation of water and loss of turgor at infected site

Figure 8. Two models describing the mechanistic basis of a decrease in water potential during the development of an HR in a leaf.
To measure the water potential that a bacterium may encounter in the absence of a compatible or incompatible interaction, we infiltrated an *A. thaliana* leaf with the saprophyte *Ps. fluorescens* strain A506(pPProlce) and measured the ice nucleation activity of the recovered cells. We observed that A506(pPProlce) encountered significantly higher water potentials *in planta* than DC3000(pPProlce). A *hrcC* mutant of DC3000 encountered similar water potentials as A506. This finding that DC3000(pPProlce) encountered lower water potentials than both A506 or DC3000 *hrcC* suggests that DC3000 can induce changes in the plant whereas A506 and the *hrcC* mutant cannot. This result indicates that *hrcC* is needed for this active function in DC3000. *HrcC* is a component of the type III secretion apparatus which allows for the secretion of effectors into the plant cell (36). Without the secretion of the effector proteins, no recognition between the plant and bacteria can occur. Our results support the fact that DC3000 *hrcC* is no longer recognized as a pathogen.

Although all three constructs containing an *avr* gene, DC3000(pPProlce/pLH12), DC3000(pPProlce/pK48-8) and DC3000(pPProlce/pPGS002), encountered a lower water potential than did DC3000(pPProlce), the *avrRpm1* gene was consistently associated with lower water potentials than *avrRpt2* or *avrB*. This observation suggests that the *avrRpm1* gene product induces a more dramatic response in the plant than the other two. Hammond-Kosack et al. (1996) have similarly demonstrated differences among distinct *avr-R* gene product interactions, with one *avr-R* gene pair inducing a faster HR response than the other (20).

The InaZ protein proved to be a useful reporter of *in planta* water potential in these studies. Originally, we attempted to use GFP-based biosensors (3) to quantify the water potential of the *A. thaliana* apoplast; however, the low fluorescence of uninduced cells of strains DC3000 and A506 containing the proU-gfp fusion prevented differentiation of those cells from plant debris using flow cytometry. The influence of non-target environmental conditions on the activity of the InaZ proteins was evaluated using the control strains DC3000(pPNptlce), DC3000 *hrcC*(pPNptlce) and A506(pPNptlce). Because of the constitutive production of InaZ in these strains, environmentally induced changes in INA can be attributed to changes in the activity of the InaZ proteins rather than to changes in *inaZ* transcription. When exposed to lower water potentials, the control strains exhibited a slight, although not significant, change in INA; this was probably due to alterations in the cytoplasmic membrane indirectly affecting the activity of the membrane-bound InaZ proteins.
As in a previous study, a plasmid-based proU transcriptional fusion was an effective tool for measuring water potential on a microscale. In a previous study, we demonstrated that proU induction was specific to water availability and not to ion toxicity or simply osmolarity (3). In this study we verified that proU did not respond to hydrogen peroxide. This demonstration was important because hydrogen peroxide is known to increase in concentration during HR (5, 23). The use of this plasmid-based transcriptional fusion also required that the plasmid vector be maintained in planta, that is, in the absence of antibiotic selection. Previous reports showed that the pPROBE vector was stable for at least 30 generations in rich medium (30). We investigated the stability of the fusion-containing plasmid under low and high osmolarity conditions and found that it was stable under both conditions for at least 30 generations (see Appendix 1).

Although we did not observe population decreases between 8 h and 24 h, we did observe a decrease in cell numbers by 4 h after inoculation. The water potentials encountered by the cells at this time were not low enough to cause the decrease in the populations in the initial time period. The decrease during these 4 h may be due to the presence of hydrogen peroxide, as indicted by a previous report that correlated an increase in hydrogen peroxide with a decrease in populations during this period following inoculation (5). Therefore, the conditions impacting the dynamics of the populations during the initial 4 h may be distinct from those affecting their subsequent dynamics.

In summary, these studies demonstrate that DC3000 cells encountered lower water potentials during the HR than during pathogenesis. In fact, during pathogenesis, DC3000 encountered water potentials that were equivalent to those that enhanced growth in culture, suggesting that during pathogenesis bacteria can actively modify their environment to favor growth. On the other hand, during HR, DC3000 encountered water potentials that were sufficiently low as to hinder cell division, although not to cause cell death. This finding is consistent with the population dynamics in planta during the HR. The lower water potentials encountered by cells during HR may be one contributing factor for the lower populations observed days after inoculation.

Acknowledgements
We thank the following for providing strains and plasmids: Sheng Yang He (DC3000 hrcC), Jean Greenberg (DC3000), Steve Lindow (A506) and Andrew Bent (pLH12, pLH12Ω, pK48-8, pPGS002). We also thank Andrew Bent for Arabidopsis thaliana seeds. Finally, we
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References


susceptible and immune cotton leaves and the local resistant response to colonies in immune leaves. Physiol. Plant Pathol. 15:53-68.


Chapter 5. Expression of Constitutive and Inducible Fusions in a Range of Prokaryotes

Introduction

A tool that enables measurements of specific environmental conditions on a microscale would be invaluable for understanding the actual conditions to which microbes are exposed in a habitat. Most methods for measuring specific environmental conditions, such as temperature, solar radiation, and water availability, function on a scale much larger than a microorganism. The extent to which a cell is exposed to extremes or fluctuations in an environment is likely to condition its adaptation response, as well as directly impact its growth and survival. The environmental condition we are interested in is water availability. Water availability is critical to microorganisms, and may be a key factor influencing bacterial growth and survival in many habitats, including soil, biofilms, flower blossoms, plant surfaces, food and skin surfaces. We have fused a water deprivation-responsive promoter to a reporter gene to create a tool that measures the water potential in a microhabitat (2). We refer to this tool as a bacterial biosensor. Specifically, we used the proU promoter ($P_{proU}$) from *Escherichia coli*, which is induced by high salt concentration and by desiccation, to the reporter gene *gfp*, which encodes a green fluorescent protein (GFP). The proU operon aids in osmoadaptation by encoding a transport system for the uptake of various compatible solutes, including glycine betaine and proline. Previous studies have demonstrated that $P_{proU}$ expression in *E. coli* is proportional to the osmolarity of the growth medium (15, 22, 25). Other features of the proU promoter that make it appealing for use include that it responds rapidly to low water potentials, it maintains high levels of expression after induction, and it functions without a regulatory protein (23, 24).

Marking a specific group of microorganisms allows researchers to distinguish them from other cells or populations in ecological studies. Stable plasmids containing fusions between constitutively expressed promoters and *gfp* have been used to mark many gram-negative bacteria (7, 10). The successful marking of a bacterial strain requires that the promoter is recognized and the reporter protein is produced and functional. Several researchers have demonstrated that detectable amounts of a reporter protein were not observed in various prokaryotes due to poor expression of the reporter gene (1, 34).

There are several examples of gene transfer from one bacterial genus to another. For example, antibiotic resistance genes on plasmids and transposons are commonly
expressed in diverse organisms. The divIVA gene from Bacillus subtilis was expressed in the distantly related species E. coli (12). Similarly, a Bacillus megaterium promoter for the xylA gene was fused to the gfp gene and shown to be expressed in Staphylococcus aureus (33), and the E. coli lacZ promoter was expressed in Vibrio sp. strain S141 (36). However, it is difficult to predict if promoters will be expressed following intergenus transfer. For example, the nptII promoter was expressed in Psychrobacter sp. strain SW5H but not in Pseudoalteromonas sp. strain S91 (36). Also the lacZ promoter was expressed efficiently in certain organisms but not in others (2). The proU promoter used in this study was originally cloned from E. coli but has been shown to respond to water deprivation in Pseudomonas syringae (2). To use the proU promoter to evaluate bacterial exposure to water deprivation in a given habitat, the proU promoter needs to function in a species that is adapted to that habitat. Thus, to explore the potential for the proU-based biosensors to be used in a variety of habitats, we evaluated expression of the promoter in a variety of gram-positive and gram-negative species; we demonstrated osmoresponsiveness in most of the organisms tested. We also marked these species with lacZ-gfp and nptII-gfp fusions and characterized the resulting fluorescence, and thus detectability, of these marked strains.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains used for these studies can be found in Table 1. For routine maintenance, strains were grown on either Tryptic Soy Agar (TSA), (Microtech Scientific, Orange, CA), Nutrient Broth Yeast (NBY) (39), Luria-Bertani (LB), Nutrient Broth Agar (NA) (Microtech Scientific, Orange, CA) or Brain Heart Infusion Agar (BHIA) (Difco, Sparks, MD) at the appropriate temperature (Table 1). K medium (18), a low osmoticum medium, amended with supplemental NaCl was used for induction studies. Antibiotics used were as follows: ampicillin (50 μg/ml), chloramphenicol (30 μg/ml), erythromycin (20 μg/ml), gentamycin (20 μg/ml), kanamycin (50 μg/ml), nalidixic acid (50 μg/ml), rifampin (50 μg/ml), spectinomycin (20 μg/ml), streptomycin (20 μg/ml) and tetracycline (20 μg/ml).

Plasmids used are listed in Table 2. The broad-host-range plasmid pVSP61 and its derivatives, including pPROBE-KT', have a pVS1 origin of replication. The plasmids were transferred to strains by triparental filter matings using the helper plasmid pRK2073. A mixture of the host, donor and helper strains (1 ml per strain) was filtered through a 0.22 μm filter (5 cm diameter). Filters were placed on the appropriate medium without antibiotics and
grown overnight at the optimal growth temperature of the recipient. Strains containing the target plasmids were selected for with kanamycin. Matings to generate each recipient-plasmid combination were performed up to 3 times, if necessary. The introduction of plasmids into some strains was hindered by the lack of a distinct antibiotic resistance marker; in these cases, colony morphology was used as a screen.

The identity of the recipient strains following conjugation was confirmed by phenotypic characterizations including Gram staining (14), colony appearance and biochemical tests. Yeast Extract Mannitol (YEM) agar (40) containing Congo Red (25 μg/ml) was used to confirm the identity of *Rhizobium* sp. The identity of the other organisms was confirmed based on the results of one or more of the following biochemical tests: Blood agar, Phenylalanine agar, Simmons' Citrate agar, Triple-Sugar Iron (TSI) agar and in Methyl Red Vogas-Proskauer (MRVP) broth (14).

**GFP Quantification**

The fluorescence of GFP-expressing cells was quantified using a Beckman-Coulter (Fullerton, CA) EPICS-XL-MCL flow cytometer. The cytometer was equipped with a 15-mW, 488-nm, air-cooled argon ion laser. GFP fluorescence was collected with a 550-nm dichroic long-pass filter and a 525 ± 20-nm band-pass filter. Voltages used were 860 V for GFP fluorescence, 790 V for side scatter (SS) and 869 V for forward scatter (FS). Baseline thresholds of fluorescence, SS and FS were used to detect GFP-expressing bacterial cells. A Coulter XL System II (version 2.1) (Beckman-Coulter, Miami, FL) was used for data acquisition while the FCS Express (version 2.0) (URL http://denovosoftware.com) software was used for data analysis. Fluorescence was expressed as relative fluorescence unit (RFU). At least 25,000 cells were examined for each sample.
Table 1. Strains used in this study

<table>
<thead>
<tr>
<th>Bacterial species (strain)</th>
<th>Relevant Characteristics*</th>
<th>Growth temperature</th>
<th>Growth medium\textsuperscript{b}</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram-negative</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aeromonas hydrophilia (AHIR)</td>
<td>Km\textsuperscript{r}, Rf\textsuperscript{r}</td>
<td>28\textdegree C</td>
<td>TSA (32)</td>
<td></td>
</tr>
<tr>
<td>Agrobacterium tumefaciens (At B6)</td>
<td>Km\textsuperscript{r}, Na\textsuperscript{r}, Sm\textsuperscript{r}, Sp\textsuperscript{r}</td>
<td>28\textdegree C</td>
<td>TSA</td>
<td>S. Lindow (UC-Berkeley)</td>
</tr>
<tr>
<td>Enterobacter aerogenes</td>
<td>Ery\textsuperscript{r}</td>
<td>37\textdegree C</td>
<td>TSA</td>
<td>ATCC 13048</td>
</tr>
<tr>
<td>Erwinia amylovora (Ea8R)</td>
<td>Rf\textsuperscript{r}</td>
<td>28\textdegree C</td>
<td>TSA</td>
<td></td>
</tr>
<tr>
<td>Escherichia coli (DH5\textalpha)</td>
<td>Na\textsuperscript{r}</td>
<td>37\textdegree C</td>
<td>LA</td>
<td></td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>no resistance</td>
<td>37\textdegree C</td>
<td>TSA</td>
<td>ATCC 13838</td>
</tr>
<tr>
<td>Methylobacterium organophilum (SH1PK)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pantoea agglomerans (BRT98)</td>
<td>Rf\textsuperscript{r}</td>
<td>28\textdegree C</td>
<td>TSA</td>
<td>(4)</td>
</tr>
<tr>
<td>Pseudomonas fluorescens (A506)</td>
<td>Cm\textsuperscript{r}, Ery\textsuperscript{r}, Rf\textsuperscript{r}, Sm\textsuperscript{r}, Sp\textsuperscript{r}</td>
<td>28\textdegree C</td>
<td>TSA</td>
<td>(20)</td>
</tr>
<tr>
<td>Pseudomonas putida (LH0001)</td>
<td>Cm\textsuperscript{r}, Ery\textsuperscript{r}, Na\textsuperscript{r}, Rf\textsuperscript{r}, Sm\textsuperscript{r}, Sp\textsuperscript{r}, derived as Rf mutant of mt-2</td>
<td>28\textdegree C</td>
<td>TSA</td>
<td>L. Halverson (Iowa State University)</td>
</tr>
<tr>
<td>Rhizobium etli (KIM5S)</td>
<td>Ery\textsuperscript{r}, Na\textsuperscript{r}, Sm\textsuperscript{r}, Sp\textsuperscript{r}</td>
<td>28\textdegree C</td>
<td>TSA</td>
<td>(3)</td>
</tr>
<tr>
<td>Rhizobium meliloti (102FB4a)</td>
<td>(no resistance)</td>
<td>28\textdegree C</td>
<td>TSA</td>
<td>S. Lindow (UC-Berkeley)</td>
</tr>
<tr>
<td>Salmonella typhimurium (ST1R)</td>
<td>Ery\textsuperscript{r}, Rf\textsuperscript{r}, Sm\textsuperscript{r}, Sp\textsuperscript{r}</td>
<td>37\textdegree C</td>
<td>TSA</td>
<td>(32)</td>
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<tr>
<td>Vibrio natrigenes</td>
<td>Sm\textsuperscript{r}, Sp\textsuperscript{r}</td>
<td>28\textdegree C</td>
<td>BHIA</td>
<td>ISU Microbiology Culture Collection</td>
</tr>
<tr>
<td>Xanthomonas maltophilia (BP1)</td>
<td>Cm\textsuperscript{r}, Ery\textsuperscript{r}, Km\textsuperscript{r}, Gm\textsuperscript{r}, Sm\textsuperscript{r}, Sp\textsuperscript{r}</td>
<td>28\textdegree C</td>
<td>TSA</td>
<td>(43)</td>
</tr>
<tr>
<td><strong>Gram-positive</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus subtilis (AS502)</td>
<td>Rf\textsuperscript{r}, Sm\textsuperscript{r}, Sp\textsuperscript{r}</td>
<td>37\textdegree C</td>
<td>TSA</td>
<td>(35)</td>
</tr>
<tr>
<td>Clavibacter michiganensis (GH2390)</td>
<td>Na\textsuperscript{r}, Rf\textsuperscript{r}</td>
<td>28\textdegree C</td>
<td>NBY</td>
<td>(4)</td>
</tr>
<tr>
<td>Mycobacterium smegmatis</td>
<td>Na\textsuperscript{r}</td>
<td>37\textdegree C</td>
<td>NA</td>
<td>ISU Microbiology Culture Collection</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>Na\textsuperscript{r}, Sm\textsuperscript{r}</td>
<td>37\textdegree C</td>
<td>TSA</td>
<td>ATCC 25923</td>
</tr>
<tr>
<td>Streptococcus thermophilus</td>
<td>Na\textsuperscript{r}</td>
<td>37\textdegree C</td>
<td>TSA</td>
<td>ISU Microbiology Culture Collection</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Ap\textsuperscript{r}, ampicillin resistance; Cm\textsuperscript{r}, chloramphenicol resistance; Ery\textsuperscript{r}, erythromycin; Gm\textsuperscript{r}, gentamycin; Km\textsuperscript{r}, kanamycin resistance; Na\textsuperscript{r}, nalidixic acid resistance; Rf\textsuperscript{r}, rifampin resistance; Sm\textsuperscript{r}, streptomycin resistance; Sp\textsuperscript{r}, spectinomycin resistance; Tc\textsuperscript{r}, tetracycline resistance.; Antibiotic resistances in bold were used for selection.

\textsuperscript{b} TSA, tryptic soy agar (Microtech Scientific, Orange, CA); NBY, nutrient broth yeast agar (39); LA, luria agar (27); NA, nutrient agar (Microtech Scientific, Orange, CA); and BHIA, brain heart infusion agar (Difco, Sparks, MD)
Table 2. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Relevant Characteristics</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pPROBE-KT</td>
<td>Broad-host-range plasmid, Km(^r), MCS upstream of promoterless gfp gene</td>
<td>(28)</td>
</tr>
<tr>
<td>pVSP61</td>
<td>Broad-host-range cloning vector containing the lacZ promoter, Km(^r)</td>
<td>(17, 21, 38)</td>
</tr>
<tr>
<td>pVSP61-Lac</td>
<td>pVSP61 without P(<em>{\text{lacZ}}) (HindIII-P(</em>{\text{Pvull}}) deletion)</td>
<td>This study</td>
</tr>
<tr>
<td>pV'Green</td>
<td>pVSP61-Lac containing the promoterless gfp gene from pGreenTIR (29) in the EcoRI site</td>
<td>This study</td>
</tr>
<tr>
<td>pRK2073</td>
<td>Tra(^r), Mob(^r), Sm(^r), Sp(^r)</td>
<td>(6)</td>
</tr>
<tr>
<td>pPPProGreen</td>
<td>pPROBE-KT(^r) containing a proU-gfp fusion, Km(^r)</td>
<td>(2)</td>
</tr>
<tr>
<td>pVLacGreen</td>
<td>pV'Green containing a lacZ-gfp fusion, Km(^r)</td>
<td>(2)</td>
</tr>
<tr>
<td>pVNptGreen</td>
<td>pV'Green containing a 364-bp SalI fragment with P(_{\text{nptII}}) from Tn5 (5, 26) cloned upstream from the gfp gene</td>
<td>This study</td>
</tr>
</tbody>
</table>

Characterization of GFP expression on solid medium

The effect of NaCl on P\(_{\text{proU-gfp}}\), P\(_{\text{lacZ-gfp}}\) and P\(_{\text{nptII-gfp}}\) was determined using cells on solid K medium amended with 0, 150, or 300 mm NaCl. Cultures were grown at optimal growth temperatures (Table 1). Two days of growth were required before analysis for A. tumefaciens, E. aerogenes, E. amylovora, E. coli, P. agglomerans, S. typhimurium, and V. natrigens; 3 days for Ps. fluorescens, Ps. putida and R. meliloti; and 4 days for M. organophilum, C. michiganensis and S. aureus. Several strains, including A. hydrophilia, K. pneumoniae, R. etli, X. maltophilia, B. subtilis, M. smegmatis and S. thermophilus, were not tested due to the attempts to introduce the plasmids by conjugation being consistently unsuccessful. Cells were resuspended in K broth directly before analysis.

Results

Comparison of fluorescence levels for proU-gfp, nptII-gfp and lacZ-gfp

Following the introduction of the fusions proU-gfp, nptII-gfp and lacZ-gfp into a diversity of strains, the fluorescence of the cells indicated that there was a strong strain specificity affecting the level of expression of each fusion (Fig. 1). proU-gfp expression in the absence of elevated osmolarity consistently resulted in less than 30 RFU. Strains M. organophilum, Ps. fluorescens, Ps. putida and E. amylovora exhibited low levels of fluorescence (<1 RFU), whereas relatively high levels were seen in S. aureus (25.1 ± 4.8 RFU) and P. agglomerans (18.7 ± 1.2 RFU). lacZ-gfp expression was not detectable in M. organophilum, Ps. fluorescens or Ps. putida (Fig. 1B), but in the remaining strains, it ranged from 6.2 ± 0.3 RFU (A. tumefaciens) to 149.8 ± 8.9 RFU (P. agglomerans). The plasmid...
pVNptGreen was not successfully transferred into *M. organophilum* or *V. natrigens*. Expression of *nptII-gfp* was much higher than *lacZ-gfp* expression in *Ps. fluorescens* and *Ps. putida* (22- to 25-fold), and slightly higher in *E. aerogenes* and *A. tumefaciens* (1.3- and 1.9-fold) (Fig. 1B and 1C). *lacZ-gfp* and *nptII-gfp* were expressed at similar levels in *C. michiganensis*, whereas *lacZ-gfp* expression was 1.6- to 4.2-fold higher than *nptII-gfp* expression in the 5 remaining strains (Fig. 1B and 1C). Among the strains, *nptII-gfp* expression was the highest in *E. amylovora* (170.4 ± 15.9 RFU).

**Effect of NaCl concentration on the fluorescence of *lacZ-gfp*-containing strains**

The fluorescence of nine of the twelve strains was less than two-fold greater in the presence of 300 mM NaCl than in 0 mM NaCl (Table 3), indicating that osmolarity had relatively little influence on *P_lacZ* expression or GFP fluorescence. The fluorescence of two of the remaining strains, *E. aerogenes* and *C. michiganensis*, was directly related to the NaCl concentration, with an approximately 4-fold greater level in the presence of 300 mM NaCl (Table 3). *V. natrigens* was unique in exhibiting > 4-fold higher fluorescence levels in the presence of each 150 mM NaCl and 300 mM NaCl (Table 3).

**Effect of NaCl concentration on the fluorescence of *nptII-gfp*-containing strains**

In the presence of 300 mM NaCl, most strains containing the *nptII-gfp* fusion exhibited fluorescence levels that were less than 2-fold greater than those at 0 mM NaCl, with two strains (*Ps. fluorescens* and *S. aureus*) exhibiting up to 3.2-fold greater levels (Table 3). These data indicate that NaCl has relatively little impact on *nptII* promoter expression or on GFP fluorescence.

**Effect of NaCl concentration on *proU-gfp* expression**

Three major trends of induction were observed for strains containing the *proU-gfp* fusion (Fig. 3). The first trend was exhibited by strains *M. organophilum, E. amylovora, S. aureus*, and *A. tumefaciens*, and involved an induction ratio of less than two at 300 mM NaCl (Fig. 2A). The strains *M. organophilum* and *S. aureus* grew poorly on solid K medium; however, when grown on media that supported ample growth (NA and TSA), the *proU-gfp-*
Figure 1. Fluorescence of various bacterial strains containing the fusions A) proU-gfp, B) lacZ-gfp and C) nptII-gfp on K medium amended with 0 mM NaCl. Values represent the mean ± standard error of the mean for n=3 samples. These data are representative of two independent studies. NA = not available for testing, RFU = relative fluorescence units.
containing *M. organophilum* and *S. aureus* strains exhibited similarly low levels of fluorescence at all NaCl concentrations examined. A second trend was exhibited by strains *P. agglomerans*, *Ps. fluorescens*, *Ps. putida*, *S. typhimurium* and *E. aerogenes* and involved a larger difference in the level of induction between 150 and 300 mM NaCl than between 0 and 150 mM (Fig. 2B). This group exhibited a 3- to 6-fold induction level by 150 mM NaCl and an 8- to 15-fold induction level by 300 mM NaCl with the exception of *S. typhimurium*, which exhibited a 30-fold increase at 300 mM NaCl. The third trend was exhibited by *E. coli*, *C. michiganensis*, *V. natrigens* and *R. meliloti*, all of which exhibited a 7- to 13-fold induction by 150 mM NaCl, and a 15- to 18-fold induction at 300 mM NaCl, all except *V. natrigens* (Fig 2C). *V. natrigens* exhibited a 25-fold increase at 300 mM NaCl. These data reflect the results of two independent studies.

Table 3. Effect of NaCl on fluorescence of lacZ-gfp- and nptII-gfp-containing strains. The induction ratio was the mean fluorescence of the cells grown with the indicated NaCl concentration relative to the mean fluorescence of cells grown with 0 mM NaCl. Values represent the mean ± standard error of the mean for n=3 samples. (NA = not available for testing)

<table>
<thead>
<tr>
<th>Species</th>
<th>lacZ-gfp</th>
<th>nptII-gfp</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>150 mM</td>
<td>300 mM</td>
</tr>
<tr>
<td><em>Agrobacterium tumefaciens</em></td>
<td>0.68 ± 0.01</td>
<td>0.80 ± 0.03</td>
</tr>
<tr>
<td><em>Clavibacter michiganensis</em></td>
<td>2.25 ± 0.00</td>
<td>3.71 ± 0.38</td>
</tr>
<tr>
<td><em>Enterobacter aerogenes</em></td>
<td>2.40 ± 0.31</td>
<td>3.95 ± 0.53</td>
</tr>
<tr>
<td><em>Erwinia amylovora</em></td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>2.07 ± 0.27</td>
<td>1.73 ± 0.24</td>
</tr>
<tr>
<td><em>Methylobacterium organophilum</em></td>
<td>0.90 ± 0.17</td>
<td>2.14 ± 0.43</td>
</tr>
<tr>
<td><em>Pantoea agglomerans</em></td>
<td>1.38 ± 0.02</td>
<td>1.51 ± 0.08</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>1.15 ± 0.25</td>
<td>1.15 ± 0.04</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em></td>
<td>0.99 ± 0.09</td>
<td>1.03 ± 0.12</td>
</tr>
<tr>
<td><em>Rhizobium meliloti</em></td>
<td>1.32 ± 0.01</td>
<td>1.41 ± 0.02</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td>2.05 ± 0.28</td>
<td>2.28 ± 0.18</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>0.93 ± 0.22</td>
<td>2.12 ± 1.07</td>
</tr>
<tr>
<td><em>Vibrio natrigens</em></td>
<td>6.05 ± 3.91</td>
<td>20.42 ± 2.88</td>
</tr>
</tbody>
</table>
Figure 2. Osmoinduction of proU-gfp in various organisms exposed to 0, 150 and 300 mM NaCl. Strains exhibited a spectrum of induction patterns including A) little to no induction, B) a larger difference in induction between 150 and 300 mM than between 150 mM and 0 mM NaCl, and C) a larger difference in induction between 0 and 150 mM than between 150 mM and 300 mM NaCl. Induction was calculated as the ratio of the mean fluorescence of the cells at a given NaCl concentration and the mean fluorescence of cells grown with 0 mM NaCl. Values represent the mean ± standard error of the mean for n = 3 samples.
Discussion

We demonstrated the ability to constitutively mark a variety of gram-negative and gram-positive organisms. Marking bacterial cells and populations with a gfp fusion can allow the detection of individual bacterial cells in a habitat (11, 13, 19), as well as promote their detection in situ using fluorescence microscopy (9, 31, 42). This is not the case with the typical antibiotic resistance markers. The extent of fluorescence exhibited by lacZ-gfp- and nptII-gfp-expressing bacteria varied among strains. This suggests strain variability in promoter expression and indicates the need to identify the optimal promoter for each strain. For example, *Ps. fluorescens* and *Ps. putida* did not exhibit detectable fluorescence with the lacZ-gfp fusion, but did with the nptII-gfp fusion. The remaining strains all exhibited detectable fluorescence with each fusion, but differed in terms of which fusion, lacZ-gfp or nptII-gfp, conferred greater fluorescence.

In this study, we also demonstrated that the proU-gfp fusion is osmoregulated in a diversity of species. Consequently, this fusion can be used as a reporter of water availability in diverse habitats by introducing it into species adapted to those habitats. The proU promoter was not osmoreponsive in *S. aureus*, *M. organophilum*, *E. amylovora*, or *A. tumefaciens*. The fluorescence of *S. aureus* containing lacZ-gfp and nptII-gfp demonstrates that proper GFP folding occurs in this strain. Proper folding of GFP into the three dimensional structure involves the processes cyclization of the chromophore and oxidization of the cyclized chromophore. Without proper folding of the protein, fluorescence yield can either be reduced or non-existent in some strains. The visible fluorescence in *S. aureus* with lacZ-gfp and nptII-gfp is supported by other reports demonstrating the effective use of GFP as a reporter protein in *S. aureus* (33). The proU promoter in *S. aureus* has a relatively high basal level of expression in the absence of NaCl suggesting that the promoter is recognized in this organism; however, the proU promoter is not osmoregulated in *S. aureus*. The basal fluorescence for proU-gfp was low for *M. organophilum*, *E. amylovora*, and *A. tumefaciens*, indicating that the promoter may be poorly recognized in these strains. The lack of fluorescence of these strains when they contained proU-gfp was not due to improper GFP folding based on their significant fluorescence when they contained the lacZ-gfp or nptII-gfp fusions. Another researcher also observed poor or undetectable proU recognition in *E. amylovora* (J. Norelli, personal communication). Two distinct patterns of induction were observed in the remaining 9 strains. For *P. agglomerans*, *Ps. fluorescens*, *Ps. putida*, *S. typhimurium* and *E. aerogenes*, a greater increase in fluorescence occurred between 150
and 300 mM NaCl than between 0 and 150 mM NaCl, suggesting a possible logarithmic increase in expression with increasing NaCl. On the other hand, a linear induction pattern was seen for the strains *E. coli*, *C. michiganensis*, *V. natrigens* and *R. meliloti* in this range of NaCl concentrations.

Some strains such as *Ps. fluorescens* and *Ps. putida* exhibited particularly low fluorescence levels regardless of the fusion or inducing conditions. The low levels of fluorescence may be due to dimerization of GFP which reduces absorption (41), improper GFP folding (8), or the reducing environment in the cytoplasm (16). The fluorescence level of *Ps. fluorescens* and *Ps. putida* containing lacZ-gfp was too low for effective strain detection in environmental samples; therefore, the nptII-gfp fusion would be a better construct for marking these strains. We previously reported lower fluorescence levels for all three fusions in *Ps. syringae pv. syringae* when compared to *E. coli* and *P. agglomerans* (2).

In general, elevated NaCl concentrations up to 300 mM NaCl did not alter *P* npW or *P* lacZ expression or GFP fluorescence. Therefore, the nptII-gfp and lacZ-gfp constructs can be used as controls to evaluate the effect of other environmental conditions on GFP activity. The use of such controls is necessary to account for non-target effects on cellular fluorescence, and thus to accurately attribute increases in the fluorescence of proU-gfp-containing strains to limitations in water availability. For example, GFP activity has been shown to be hindered by extremes in pH, temperature, and sunlight (37)(C. Chen, personal communication).

We have demonstrated the ability to mark a wide variety of microorganisms with either lacZ-gfp or nptII-gfp. For some applications, such as monitoring an organism in the soil or on plants, GFP production may confer a fitness cost on the organism; therefore, a fusion producing less GFP may be preferable. However, fusions conferring fluorescence at levels above the detection limit of a flow cytometer or microscope are necessary for applications using this techniques. For example, if *Ps. putida*(pVLacGreen) cells were inoculated onto a leaf and then recovered and analyzed using a flow cytometer, the GFP-expressing cells could not be distinguished from plant debris or non-fluorescing cells due to the low levels of fluorescence. We also demonstrated that the proU promoter was osmoresponsive in a variety of gram-positive and gram-negative bacteria. The fact that the species examined represent organisms from a variety of habitats indicated that proU-based water deprivation-responsive bacterial biosensors could be constructed for examining the availability of water in diverse bacterial habitats.
Acknowledgements

We thank Claudia Lemper and Jeness Peterson for the construction of the plasmids pVSP61-Lac and pV’Green. We thank the following for providing strains: Robert Andrews (B. subtilis), Robert Hubert (E. aerogenes, K. pneumoniae, M. smegmatis, S. aureus, S. thermophilus and V. natriegens), Steve Lindow (A. hydrophila, A. tumefaciens, M. organophilum, Ps. fluorescens, R. meliloti and X. maltophilia), and Charles Block (C. michiganensis). We also thank Lindsay Schulz for her assistance with the experiments.

References


General Conclusions and Future Directions

We have been able to measure the water deprivation encountered by microorganisms both in and on aerial leaves. We developed a water-responsive bacterial biosensor that allows us to measure the amount of water limitation bacteria encounter in the environment. We fused the proU promoter to a reporter gene, either gfp or inaZ, and placed the fusion on a broad-host-range plasmid. The bacterial biosensor was very sensitive, responded to water deprivation in a quantitative manner and had a broad range of detection. The multi-copy plasmid was determined to be stable for at least 30 generations and allowed for amplification of the response to water deprivation. We observed osmoreponsiveness in a variety of microorganisms including Vibrio natrigenes, Pseudomonas syringae and Clavibacter michiganensis. This supports previous literature stating that the proU promoter does not require a specific regulatory element. We were able to measure proU-gfp expression in a saprophyte, Pantoea agglomerans BRT98, on bean leaves when exposed to various RH levels. Little water deprivation was encountered by BRT98. In fact, growth in culture indicates that this level of water deprivation does not kill BRT98 cells, but does increase their lag time and reduce their growth rate. We also investigated the driving factor for the bacterial population dynamics reported by previous researchers during pathogenesis and HR. Using an InaZ-based biosensor in Ps. syringae pv. tomato strain DC3000 in Arabidopsis thaliana, we demonstrated that DC3000 encountered lower water potentials during HR than during pathogenesis. Based on the effect of low water potential on DC3000 growth and survival in culture, the water potentials encountered by cells during pathogenesis should have enhanced the growth rate of DC3000, whereas the water potentials encountered during HR should have inhibited DC3000 growth, but not killed it. We also measured the amount of water deprivation encountered by a saprophyte in the A. thaliana leaf and demonstrated that saprophytes encounter significantly higher water potentials than pathogens. These results suggest that pathogens can actively manipulate the internal spaces of the plant to create a more favorable environment for growth.

Although we were able to measure the water potential encountered by BRT98 on leaf surfaces and DC3000 in leaves, there are two limitations of the bacterial biosensors that were constructed. The first limitation was the ineffectiveness of GFP as a quantitative reporter protein under field conditions. Specifically, during the field studies we observed a dramatic decrease in the fluorescence of the biosensor cells. Subsequent studies
performed by others in the laboratory concluded that GFP is sensitive to sunlight (Chen and Beattie, unpublished data); therefore, successful field studies would require minimizing bacterial exposure to sunlight, such as by covering the plants. However, a cover could alter the conditions on the aerial leaf surface therefore altering the environmental conditions that are being evaluated. The second limitation was the potential detrimental effect of environmental stress on transcription within the cell under conditions in which metabolic activity is hindered. Specifically, expression of the promoter that is central to a transcription-based bacterial biosensor may be decreased by factors influencing general metabolic activity such as severe environmental stresses, and thus may not report on the actual target environmental conditions.

For several studies, an InaZ-based biosensor was used because DC3000 cells labeled with GFP were unable to be distinguished from plant debris following recovery of the cells by homogenization because of the low level of fluorescence. Although there are several advantages of InaZ as a reporter gene, such as its sensitivity and wide range of activity, there are several limitations. First, the water potentials encountered can only be estimated for the entire population rather than on a cell by cell basis. Second, assays used to quantify ice nuclei tend to be very laborious. And third, ice activity is very temperature dependent. Temperatures greater than 24°C can reduce ice activity.

Initially, we wanted to examine the water deprivation encountered by a saprophyte and pathogen on the aerial leaf surface. We successfully measured the water potential encountered by a saprophyte, \textit{P. agglomerans}, on the aerial leaf surface (as mentioned above). Our efforts to quantify water potentials encountered by the pathogen \textit{Ps. syringae} pv. \textit{syringae} strain B728a were not successful. Compared to saprophytes, B728a populations are generally found to be higher inside the leaf; therefore, this microorganism may be able to avoid environmental factors by moving inside the leaf. Water relation comparison between saprophytes and pathogens is still a very exciting avenue to pursue in the future. We observed fluorescence of B728a(pPProGreen) cells in a larger area on bean leaves when compared to BRT98(pPProGreen) cells using epifluorescence microscopy. This observation suggests that a greater number of B728a cells were exposed to water limitation on leaf surfaces. Therefore, determining the water potential B728a compared to BRT98 encounters on leaf surfaces might be a first step in understanding the differences in environmental stresses encountered between a pathogen and a saprophyte. To evaluate B728a water deprivation on leaves, we proposed the use of a second marker to separate
the B728(pPProGreen) cells from background debris. We investigated a wide variety of GFP derivatives and dyes that could serve as second markers. One dye, PKH26, which binds to cell membranes, looks promising for future studies, at least in the laboratory. Another possibility that should be investigated is to identify a monoclonal antibody for B728a and label it with a fluorescent tag. Although this strategy is promising, our initial objective was to create a fluorescent marker that can be transferred to multiple microorganisms. To amplify the fluorescence of the uninduced proU-gfp fusion-containing B728a cells, we could also introduce the plasmid pVProGreen containing the proU-gfp fusion as was discussed in Chapter 2. pVProGreen does not have the downstream silencer region of the proU promoter; therefore, the basal level of expression may be high enough to separate the labeled B728a cells from the non-fluorescing cells and plant debris. This deletion could, however, reduce the range of detection of the biosensor.

Water deprivation is not the only environmental stress that may impact bacterial survival and growth on aerial leaf surfaces. It would be fascinating to investigate what role other environmental factors including UV radiation and hydrogen peroxide play in the development of bacterial populations on leaves. Biofilm or aggregate formation on leaf surfaces is also an important avenue of study. As our results indicate, biofilms may reduce bacterial exposure to water stress. Future studies may show more detail on water stress as well as if exposure to other environmental factors is reduced in biofilms.

We have reported a very exciting difference in water relations encountered by bacteria during HR versus pathogenesis. We have proposed two models to explain the decreased water availability encountered by bacteria during HR. In the first model, bacteria encounter water deprivation due to matric stress, i.e. the lack of water; however, in the second model, bacteria encounter high osmolarity. The biosensor we constructed does not differentiate between the two models because the proU promoter responds to both osmotic and matric stress. The actual conditions cells encounter during the progression of HR and pathogenesis would greatly enhance our understanding of this process. Future work could include differentiating between the two proposed models by using biosensors with fusions specific to matric or osmotic stress.

We demonstrated that the proU promoter is osmoreponsive in a variety of gram-negative and gram-positive bacteria including Ps. fluorescens, Enterobacter aerogenes, Clavibacter michiganensis and Rhizobium meliloti. Therefore, the proU-gfp fusion could be used to investigate water deprivation encountered by bacteria in a variety of environments.
The water-responsive bacterial biosensor could be used to measure the amount of water deprivation encountered by bacteria in soil and food. For example, a previous researcher used corn mutants that differed in their cuticle composition to demonstrate that the cuticle of the leaf influences bacterial colonization (Marcell, L. M. and G. A. Beattie, 2002, MPMI 15:1236-1244). It may be possible that the nature of the crystals influences the extent of evaporation of the water on the leaf surface, and thus influences how favorable the environment is for bacterial survival and growth. The water-responsive bacterial biosensor could be used to test this hypothesis. In addition to future studies on water availability with these bacterial biosensors, studies directed at generating a broader picture of the environmental stresses encountered by bacteria on leaf surfaces and the impact of these stresses on cells is very important for the development of phyllosphere microbiology including for the improvement of disease management.
Appendix 1: Plasmid Stability

In our research, we have measured gene expression *in vitro* and also on bean leaf surfaces. The ability of a strain to maintain a plasmid during growth is critical for the effective use of our plasmid-based bacterial biosensors. Plasmid stability is not an issue when gene expression measurements are conducted *in vitro* because antibiotic selection can be maintained. There are, however, many experiments such as experiments *in planta* in which the addition of antibiotics is not possible. Two plasmids, pVSP61(2, 3, 6) and pPROBE-KT (4), harbored the fusions of interest for our studies. Although the plasmid pPROBE is known to be stable in rich medium (Luria-Bertani) for more than 30 generations (4), low nutrient media are more relevant to the oligotrophic habitat of a leaf surface than rich media. Plasmids that are not essential for bacterial survival may be lost due to the additional fitness cost they confer on the organism. To test if low nutrients or low water potentials reduced plasmid stability, we examined plasmid stability in low osmoticum, low nutrient medium ½-21C (1, 5) in the absence of NaCl (Table 1) and in the presence of NaCl (Table 2). We report that the plasmids were stable for the test period in ½-21C broth and on ½-21C plates amended with NaCl.
Table 1. Stability of various plasmids in *Escherichia coli* strain DH5α, *Pseudomonas syringae* pv. *syringae* strain B728a, and *Pantoea agglomerans* strain BRT98. Strains were grown at 28°C (BRT98 and B728a) or 37°C (DH5α) in either ½-21C broth or on solid ½-21C medium.

<table>
<thead>
<tr>
<th>Strain ID</th>
<th>Plasmid stability during growth in ½-21C broth(^a)</th>
<th>Plasmid stability during growth on ½-21C plates(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Log(CFU/ml)</td>
<td>Percent Km(^r)</td>
</tr>
<tr>
<td></td>
<td>LA</td>
<td>LA + Km</td>
</tr>
<tr>
<td>BRT98 pPROBE-KT</td>
<td>9.77 ± 0.04</td>
<td>9.76 ± 0.04</td>
</tr>
<tr>
<td>BRT98 pPProGreen</td>
<td>9.89 ± 0.07</td>
<td>9.91 ± 0.10</td>
</tr>
<tr>
<td>BRT98 pVLacGreen</td>
<td>9.86 ± 0.27</td>
<td>9.90 ± 0.25</td>
</tr>
<tr>
<td>B728a pPProGreen</td>
<td>9.87 ± 0.18</td>
<td>9.84 ± 0.23</td>
</tr>
<tr>
<td>DH5α pPProGreen</td>
<td>9.12 ± 0.01</td>
<td>9.14 ± 0.02</td>
</tr>
</tbody>
</table>

\(^a\) A single colony was inoculated into 5 ml of ½-21C broth and was placed on a shaker. After 24 h, 5 μl of the culture was transferred to 5 ml of ½-21C broth and an aliquot was plated onto solid Luria-Bertani medium (LA) with and without kanamycin (50 μg/ml). This procedure was repeated 4 times. Values shown represent the mean ± standard error of the mean for \(n = 2\) samples at the final sampling. Each 24 h period was calculated to be equivalent to 10 generations; therefore, plasmids were determined to be stable for at least 40 generations in ½-21C broth.

\(^b\) A single colony was resuspended in ½-21C broth and an aliquot was plated onto solid ½-21C medium. After 24 h (DH5α or BRT98) or 48 h (B728a), a colony was resuspended in ½-21C broth and aliquots were plated onto ½-21C medium as well as onto LA with and without kanamycin (50 μg/ml). This procedure was repeated 3 times. Values shown were generated in the final sampling. Every sampling time was calculated to be equivalent to 15 generations; therefore, plasmids were stable for at least 45 generations on ½-21C plates.
Table 2. Lack of an effect of elevated osmolarity on the stability of the lacZ-gfp-containing plasmid in *Pantoea agglomerans* strain BRT98. BRT98 pVLacGreen cells were inoculated into ½-21C broth without antibiotics containing either 0, 200 or 400 mM NaCl and incubated at 28°C. Every 2 d for 10 d, 100 μl of the culture were transferred to 5 ml of fresh medium and samples were plated onto solid Luria-Bertani medium (LA) containing rifampin (50 μg/ml) or rifampin (50 μg/ml) and kanamycin (50 μg/ml). The plasmid was determined to be stable in the presence of NaCl for at least 30 generations at all NaCl concentrations tested. There were no significant differences in stability over time or among concentrations at each time point throughout the study based on Fisher’s LSD test (P < 0.05). Values represent the mean ± standard error of the mean for n = 3 samples.

<table>
<thead>
<tr>
<th>Sampling time</th>
<th>0 mM NaCl</th>
<th>200 mM NaCl</th>
<th>400 mM NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Log (CFU/ml)</td>
<td>Percent Km'</td>
<td>Log (CFU/ml)</td>
</tr>
<tr>
<td></td>
<td>LA+Rif</td>
<td>LA+Rif+Km</td>
<td>LA+Rif</td>
</tr>
<tr>
<td>2 d</td>
<td>8.21 ± 0.06</td>
<td>8.07 ± 0.08</td>
<td>7.94 ± 0.05</td>
</tr>
<tr>
<td>4 d</td>
<td>7.92 ± 0.05</td>
<td>7.94 ± 0.03</td>
<td>7.76 ± 0.05</td>
</tr>
<tr>
<td>6 d</td>
<td>8.04 ± 0.02</td>
<td>8.00 ± 0.01</td>
<td>7.85 ± 0.04</td>
</tr>
<tr>
<td>8 d</td>
<td>7.44 ± 0.31</td>
<td>7.79 ± 0.13</td>
<td>7.74 ± 0.16</td>
</tr>
<tr>
<td>10 d</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

References

Appendix 2. Comparison of Fluorescent Markers as Second Markers for GFP-Based Reporter Systems

Introduction

Since the original isolation of the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*, GFP has been used as a reporter in a wide variety of applications, including studies on gene expression, protein localization, and localization of microorganisms in their natural habitats. Some advantages of GFP are its substrate independence, stability, low toxicity, and ease of detection. For our studies, we would like to identify one or more GFP derivatives or other fluorescent markers that can function as a second marker in bacterial cells that contain a GFP fusion. Although GFP derivatives have been used to mark distinct bacterial populations, they have not been extensively examined for their use as dual, independent markers within individual bacterial cells. A dual marker system could be utilized to measure expression of two distinct genes in an individual cell or to isolate cells from a population based on two distinct markers using flow cytometry. We attempted to find a fluorescent marker that could be used as a second, independent marker in a *gfp*-expressing cell. Our criteria for this marker were that: 1) it was non-toxic to the bacterial cell, 2) it was detectable for multiple generations, and 3) it exhibited a distinct excitation and/or emission spectrum from the red-shifted variant of GFP.

A variety of GFP derivatives and fluorescent dyes have been developed which exhibit separate excitation and/or emission spectra from the wild-type GFP. Due to the non-overlapping spectra of some GFP derivatives, they may be used to label multiple targets for simultaneous detection. GFP derivatives are most often constructed by altering the three amino acids Ser-Tyr-Gly (65-67) involved in chromophore formation. These derivatives offer the same advantages as GFP when used as marker proteins. Similar to GFP derivatives, fluorescent proteins isolated from the organisms *Obelia* and *Renilla* may be used as marker proteins. Substrates that become fluorescent could also be used as cellular markers. In this case, a gene for the catalytic enzyme could be introduced into the strain of interest and a fluorescent substrate could be added prior to analysis. Fluorescent dyes that bind DNA or membranes can be employed to mark the population of interest. Lastly, fluorescent-labeled oligonucleotides and antibodies could be effective second markers. These must be tailored to each individual strain and thus are not amenable to marking many diverse strains.
Although there are a variety of GFP derivatives available, we chose three to examine: the blue fluorescent protein (BFP), Sapphire and the yellow fluorescent protein (YFP). These derivatives differ due to changes in the sequence of the chromophore (Table 1). Recently, reports have been published that involve labeling individual eukaryotic cells with multiple GFP derivatives. For example, YFP and the cyan fluorescent protein (CFP) were used to detect distinct structures in neurons and muscle tissue in mammalian cells (14). GFP derivatives have also been used to examine intra- and inter-protein interactions by exploiting the requirement that two derivatives must be in close contact (<100Å) for fluorescence resonance energy transfer (FRET) to occur. This phenomenon occurs when one derivative emits energy that can then be used to excite another. FRET has been reported between BFP and GFP in *Escherichia coli* (10, 17), and between BFP and GFP, and CFP and YFP, in mammalian cells (18).

Table 1. Maximum excitation and emission wavelengths of the fluorescent proteins used in this study.

<table>
<thead>
<tr>
<th>Derivative</th>
<th>Mutationa</th>
<th>Max. excitation</th>
<th>Max. emission</th>
<th>Reference/ Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green fluorescent protein</td>
<td>S65T, F64L</td>
<td>488 nm</td>
<td>510 nm</td>
<td>(23)</td>
</tr>
<tr>
<td>Yellow fluorescent protein</td>
<td>S65G, V68L, S72A, T203Y</td>
<td>513 nm</td>
<td>527 nm</td>
<td>(23)</td>
</tr>
<tr>
<td>Blue fluorescent protein</td>
<td>Y66H</td>
<td>380 nm</td>
<td>440 nm</td>
<td>(23)</td>
</tr>
<tr>
<td>Sapphire</td>
<td>T203I, S72A, Y145F</td>
<td>399 nm</td>
<td>511 nm</td>
<td>(23)</td>
</tr>
<tr>
<td>DsRed</td>
<td>None</td>
<td>558 nm</td>
<td>583 nm</td>
<td>Clontech, Inc, Palo Alto, CA</td>
</tr>
</tbody>
</table>

a Substitutions from the primary sequence of GFP are shown
b This red-shifted variant that exhibits a similar emission spectrum to the wild-type GFP is referred to simply as GFP in this study.

Other fluorescent proteins including those from the organisms *Obelia* and *Renilla* have been isolated. Clontech, Inc. released pDsRed that encodes the red fluorescent protein (DsRed) cloned from the IndoPacific sea anemone-relative *Discosoma* sp. Due to its spectral properties DsRed can be excited by the same wavelength that excites GFP, 488nm, but is separable from GFP based on its emission spectrum.

Other possible second markers were fluorescent substrates for reporter proteins such as β-Gal and PhoA. A population that contained a gene for one of these reporter proteins could be amended with the substrate immediately before analysis. Ideally, the substrate would be cleaved to produce a fluorescent product. A variety of fluorescent
substrates are available for β-galactosidase, including fluorescein digalactoside (FDG) (ex. max 490nm; em. max 514nm), which can be used in prokaryotes. Similarly, the substrate for alkaline phosphatase 4-methylumbelliferyl phosphate (MUP) (Molecular Probes, Inc., Eugene, OR) fluoresces (ex. max 360 nm; em. max 449nm) after cleavage by PhoA in prokaryotes.

Many fluorescent DNA and membrane-binding dyes are known and could be used as second markers. These dyes are widely used in eukaryotic systems but are rarely used in prokaryotes. For example, a variety of DNA dyes, designated Syto Red dyes (Molecular Probes, Inc., Eugene, OR), may be promising based on the manufacturer’s claim that they mark eukaryotic cells without influencing cellular viability, and that they are maintained through multiple generations (Molecular Probes, personal communication). Similarly, a membrane dye called PKH26 cell tracker dye (Sigma-Aldrich Corp., St. Louis, MO) (ex. max 551 nm and em. max 567 nm) binds to eukaryotic cell membranes and is maintained for ten generations. This dye has been used successfully in Gram-positive bacteria, including *Mycobacterium* sp. and *Bacillus* sp. (5, 19), but has not yet been tested with Gram-negative species.

The long-term goal of our research is to understand how bacteria colonize aerial leaf surfaces and how this colonization is influenced by environmental factors such as water deprivation. We have constructed bacterial biosensors that produce GFP in response to water deprivation (1), and have introduced the required *gfp*-fusion containing plasmids into bacteria that are well adapted to a leaf habitat, specifically *Pseudomonas syringae* and *Pantoea agglomerans*. Although *P. agglomerans* cells that were exposed to only low levels of water deprivation before recovery from leaves exhibited sufficient fluorescence to distinguish them from the plant debris in the sample, the *Ps. syringae* cells did not. Evaluation of the water deprivation sensed by the *Ps. syringae* cells on leaves thus requires a secondary fluorescent marker that will enable us to distinguish the *Ps. syringae* cells from plant debris and non-fluorescing cells.

The objective of this study was to identify a second fluorescent marker that can function as a continuously expressed marker in the water-responsive bacterial biosensors. This marker would allow us to distinguish biosensors cells that are not expressing GFP, or are expressing it at low levels, from non-biosensor cells. Ideally, this marker would not interfere with our ability to quantitatively relate GFP fluorescence to bacterial exposure to water stress. Here we describe our evaluation of three GFP derivatives, YFP, Sapphire, and
BFP, as well as DsRed, Syto Red #64 nucleic acid dye and the membrane dye PKH26 as second markers to label GFP-producing cells.

**Materials and Methods**

**Strains, plasmids and growth conditions**

The bacterial strains and plasmids used in this study are shown in Table 2. For routine maintenance, strains were grown on Luria-Bertani (LB) medium (15) at 37°C for *Escherichia coli* strain DH5α and 28°C for *Pantoea agglomerans* strain BRT98 and *Pseudomonas syringae pv. syringae* strain B728a. A low-osmoticum medium, one-half-strength 21C (½-21C) (8, 21), amended with NaCl was used for induction assays. ½-21C was amended with vitamin B₁ (0.0005%) for growth of DH5α. The following antibiotics and concentrations (µg/ml) were used: ampicillin (50), kanamycin (50), rifampin (50), spectinomycin (20), streptomycin (20) and tetracycline (20). Plasmids were mobilized by triparental matings using the helper plasmid pRK2073.

**Fluorescence Detection**

The fluorescence of cells grown in ½-21C broth were quantified using one of two flow cytometers, a Beckman-Coulter (Fullerton, CA) EPICS-XL-MCL or a Beckman-Coulter (Fullerton, CA) EPICS-ELITE. Excitation and emission settings for all fluorescent markers are shown in Table 3. Fluorescent bacterial cells were detected based on fluorescence, forward scatter (FS) and side scatter (SS) signals above baseline thresholds using controls for each experiment. Fluorescence, FS and SS were collected with logarithmic amplifiers. Data analysis and graphical representation were done with FCS Express (version 2.0) software (De Novo Software). At least 25,000 cells were examined for each sample. The geometric means of fluorescence and FS were calculated for all samples. Fluorescence was expressed as relative fluorescence units (rfu).
<table>
<thead>
<tr>
<th>Strain/Plasmid</th>
<th>Relevant Characteristics</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL21</td>
<td>contains T7 RNA polymerase gene under control of <em>lacUV5</em> promoter</td>
<td>Novagen, Madison, WI</td>
</tr>
<tr>
<td>DH5α</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Top10F'</td>
<td><em>lacI</em>, <em>Tn10</em>, <em>Tc</em>, <em>Sm</em></td>
<td>Gibco-BRL, Rockville, MD</td>
</tr>
<tr>
<td>Pantoea agglomerans</td>
<td>Rf</td>
<td>Invitrogen Corp., Carlsbad, CA</td>
</tr>
<tr>
<td>BRT98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudomonas syringae pv. syringae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B728a</td>
<td>Rf</td>
<td>(3)</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUC18</td>
<td>Cloning vector with CoIE1 replicon, Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(25)</td>
</tr>
<tr>
<td>pUC19</td>
<td>Cloning vector with CoIE1 replicon, Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(25)</td>
</tr>
<tr>
<td>pPD16.43</td>
<td>pUC19 derivative, Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(6)</td>
</tr>
<tr>
<td>pRSETa</td>
<td>pUC derived expression vector; N-terminal 6-His tag; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Invitrogen Life Technologies, Carlsbad, CA</td>
</tr>
<tr>
<td>pVSP61</td>
<td>Broad-host-range cloning vector containing the <em>lacZ</em> promoter, Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(11, 12, 24)</td>
</tr>
<tr>
<td>pPROBE-KT'</td>
<td>Broad-host-range cloning vector, Km&lt;sup&gt;+&lt;/sup&gt;, MCS upstream of promoterless <em>gfp</em> gene</td>
<td>(16)</td>
</tr>
<tr>
<td>pVSP61-Lac</td>
<td>pVSP61 without <em>P</em>&lt;sub&gt;lacZ&lt;/sub&gt; (<em>HindIII-PvuII</em> deletion)</td>
<td>This study</td>
</tr>
<tr>
<td>pRK2073</td>
<td><em>Tn&lt;sup&gt;+&lt;/sup&gt;</em>&lt;sub&gt;10&lt;/sub&gt;, <em>Mob&lt;sup&gt;+&lt;/sup&gt;</em>&lt;sub&gt;*, Sm&lt;/sub&lt;sup&gt;+&lt;/sup&gt;, Sp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(4)</td>
</tr>
<tr>
<td>pPNptGreen</td>
<td>pPROBE-KT containing the <em>Tn5 nptII</em> promoter fused to <em>gfp</em></td>
<td>(1)</td>
</tr>
<tr>
<td>pPProGreen</td>
<td>pPROBE-KT&lt;sup&gt;+&lt;/sup&gt; containing a <em>proU-gfp</em> fusion, Km&lt;sup&gt;+&lt;/sup&gt;</td>
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</tr>
<tr>
<td>pVLacGreen</td>
<td>pV&lt;sup&gt;+&lt;/sup&gt;Green containing a <em>lacZ-gfp</em> fusion, Km&lt;sup&gt;+&lt;/sup&gt;</td>
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</tr>
<tr>
<td>pDsRed</td>
<td><em>P&lt;sub&gt;lacZ-DsRed&lt;/sub&gt;</em> on pUC18, Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Clontech, Inc., Palo Alto, CA</td>
</tr>
<tr>
<td>pEBFP</td>
<td>pPD16.43 containing *P&lt;sub&gt;lacZ-bfp&lt;/sub&gt; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Clontech, Inc., Palo Alto, CA</td>
</tr>
<tr>
<td>pEYFP</td>
<td>pPD16.43 containing *P&lt;sub&gt;lacZ-yfp&lt;/sub&gt; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Clontech, Inc., Palo Alto, CA</td>
</tr>
<tr>
<td>pMS2B6</td>
<td><em>pilA-phoA</em> fusion on pUC18, Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(22)</td>
</tr>
<tr>
<td>pSapphire</td>
<td><em>P&lt;sub&gt;T7-sapphire&lt;/sub&gt;</em> on pRSETa, Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Aurora Corp., Madison, WI</td>
</tr>
<tr>
<td>pPLRProGreen&lt;sup&gt;b&lt;/sup&gt;</td>
<td>pPROGreen containing a <em>PvuII-Stul P&lt;sub&gt;lacZ&lt;/sub&gt;</em></td>
<td>This study</td>
</tr>
<tr>
<td>pPLRNptGreen&lt;sup&gt;b&lt;/sup&gt;</td>
<td>pPNptGreen containing a <em>PvuII-Stul P&lt;sub&gt;lacZ&lt;/sub&gt;</em></td>
<td>This study</td>
</tr>
<tr>
<td>pVLacRed</td>
<td>pVSP61-Lac containing a <em>PvuII-Stul P&lt;sub&gt;lacZ&lt;/sub&gt;</em></td>
<td>This study</td>
</tr>
</tbody>
</table>

<sup>a</sup> Ap<sup>+</sup>, ampicillin resistance; Km<sup>+</sup>, kanamycin resistance; Ni<sup>+</sup>, nalidixic acid resistance; Rf<sup>+</sup>, rifampin resistance; Sm<sup>+</sup>, streptomycin resistance; Sp<sup>+</sup>, spectinomycin resistance; Tc<sup>+</sup>, tetracycline resistance

<sup>b</sup> The *P<sub>lacZ-DsRed</sub>* fusion from pDsRed was inserted in the opposite direction of the existing *gfp* fusions; the orientation was confirmed by sequencing.
### Table 3. Flow cytometer settings for dual marker studies.

<table>
<thead>
<tr>
<th>Secondary marker</th>
<th>GFP fluorescence</th>
<th>Secondary fluorescence</th>
<th>Instrument</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Excitation</td>
<td>Emission&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Excitation</td>
</tr>
<tr>
<td>BFP&lt;sup&gt;b&lt;/sup&gt;</td>
<td>488 nm</td>
<td>525-535 BP</td>
<td>350 nm</td>
</tr>
<tr>
<td>DsRed</td>
<td>488 nm</td>
<td>510-520 BP</td>
<td>488 nm</td>
</tr>
<tr>
<td>Sapphire</td>
<td>488 nm</td>
<td>525-535 BP</td>
<td>408 nm</td>
</tr>
<tr>
<td>YFP</td>
<td>488 nm</td>
<td>510-520 BP</td>
<td>488 nm</td>
</tr>
<tr>
<td>PhoA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>488 nm</td>
<td>525-535 BP</td>
<td>350 nm</td>
</tr>
<tr>
<td>PKH26</td>
<td>488 nm</td>
<td>510-520 BP</td>
<td>488 nm</td>
</tr>
<tr>
<td>Syto Red #64</td>
<td>488 nm</td>
<td>525-535 BP</td>
<td>633 nm</td>
</tr>
</tbody>
</table>

<sup>a</sup> DL = dichroic longpass; BP = bandpass

<sup>b</sup> A UV blocker was used (<350 nm)

### Characterization of fluorescence in culture

For characterization of GFP-expressing cells that also produced either YFP, BFP, Sapphire or DsRed, cells were grown in ½-21C broth amended with 0, 50, 100, 200, 400 or 600 mM NaCl for 48 h at either 28°C or 37°C. Cultures were diluted to approximately $10^7$ cells/ml and were analyzed using flow cytometry. To stain cells with the nucleic acid Syto Red stain #64 (Molecular Probes, Inc., Eugene, OR), 1 ml of cells at a concentration of $10^9$ cells/ml was washed twice in 10 mM phosphate buffer. Syto dye was added at a final concentration of 0.01 mM and cells were incubated at room temperature for 30 minutes. The stained cells were pelleted and diluted to $10^7$ cells/ml in ½-21C broth. Cultures were examined over a 48 h time period. For staining cells with the cell membrane dye PKH26 (Sigma-Aldrich Corp., St. Louis, MO), overnight cultures were diluted to $10^7$ cells/ml, and cells were washed twice with 10 mM phosphate buffer and resuspended in 10 µl of phosphate buffer. 100 µl of diluent C (Sigma Corp.) was added to the cells. PKH26 dye was added to the cells suspensions at a final concentration of 10 µM/µl and the cells were incubated for 3 minutes at room temperature. The reaction was stopped using 20% BSA. Cells were pelleted and resuspended in ½-21C broth and were examined over a 48 h period. For phoA-expression analysis, cells were grown in ½-21C broth amended with NaCl at 28°C for 48 h. Cells were pelleted and resuspended in 10 mM phosphate buffer and the optical density ($OD_{600}$) was adjusted to 0.3. Cells (2 ml) were transferred to a new tube and 2 ml of 10 mM phosphate buffer containing 0.04 M glycine and 25 mM MUP (4-Methylumbelliferyl phosphate, Molecular Probes, Inc.) (pH 8) was added. The mixture was incubated at 37°C for 30 minutes and analyzed.
Evaluation of Toxicity

The effect of NaCl concentration on the growth of the GFP-expressing strains carrying the second marker was assessed by growing cells in ½-21C broth, amended with NaCl as described above. After 48 h, growth was assessed based on turbidity or cell counts on solid Luria-Bertani (LB) medium.

Results and Discussion

Yellow fluorescent protein

We introduced a plasmid containing a constitutively-expressed lacZ-yfp fusion into E. coli in the presence or absence of either the proU-gfp or lacZ-gfp fusion. The co-expression of the two GFP derivatives in a cell did not reduce the size of the population that developed in ½-21C medium in 48 h, when compared to the size of the population of strains expressing only one GFP derivative or no GFP derivative (data not shown). Therefore, co-expression of GFP and YFP was not toxic to the cells. YFP-expressing cells could be distinguished from non-fluorescent cells based on fluorescence, but approximately 25 to 30% of the YFP-expressing cells were below the threshold levels established for optimum separation of populations of YFP- and GFP-expressing strains (pEYFP, Table 4 and Fig. 1). Due to the high level of fluorescence of the GFP-expressing strains, the detection threshold allowed for the detection of the vast majority of the GFP-expressing population (pVLacGreen, Table 4 and Fig. 1).

When approximately equal cell numbers of DH5α(pVLacGreen) and DH5α(pEYFP) were combined, the two cell populations were clearly separable (pEYFP and pVLacGreen, Table 4). Approximately 50% of the cells appeared to be GFP-expressing cells, based on their assignment to quadrant 2. Similarly, 50% of the cells appeared to be YFP-expressing cells: 30% were assigned to quadrant 3 and the remainder cells were assigned to quadrant 1, as predicted. In a 1:1 mixture, the mean fluorescence of YFP- and GFP-expressing cells was similar to when cells were alone in cultures (pVLacGreen, pEYFP, and pVLacGreen and pEYFP, Table 5 and Fig. 2).

The fusion of GFP to two distinct promoters, one of which was inducible, provided a means of testing for the effect of YFP production on GFP fluorescence and cell viability at various GFP expression levels. As is illustrated in Table 5, the uninduced proU-gfp fusion (pPProGreen, 0 mM) was expressed at a higher level than the constitutively expressed lacZ-
gfp fusion (pVLacGreen), and the proU-gfp fusion was induced by NaCl (pPProGreen, 400 mM), as expected.

![Figure 1](image)

Figure 1. Density plots of E. coli DH5α cells with the fusions a) lacZ-gfp and b) lacZ-yfp. Quadrant 1 contains cells or debris with no or low levels of fluorescence. Quadrant 2 contains cells exhibiting GFP fluorescence. Quadrant 3 contains cells exhibiting YFP fluorescence. Quadrant 4 contains cells exhibiting both GFP and YFP fluorescence.

Table 4. Percentage of cells selected to distinguish GFP fluorescence from YFP fluorescence in quadrants 1-4 as described in Figure 1. Values represent the mean percent of events represented in a quadrant ± standard error of the mean (SEM) for n = 3 samples.

<table>
<thead>
<tr>
<th>Strain ID</th>
<th>Quadrant 1</th>
<th>Quadrant 2</th>
<th>Quadrant 3</th>
<th>Quadrant 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>pVLacGreen</td>
<td>2.43 ± 0.13</td>
<td>96.43 ± 0.12</td>
<td>0.30 ± 0.02</td>
<td>0.85 ± 0.02</td>
</tr>
<tr>
<td>pEYFP</td>
<td>25.07 ± 0.84</td>
<td>0.15 ± 0.01</td>
<td>73.30 ± 0.72</td>
<td>1.51 ± 0.13</td>
</tr>
<tr>
<td>pPProGreen (0mM)</td>
<td>2.40 ± 0.07</td>
<td>97.43 ± 0.07</td>
<td>0.04 ± 0.01</td>
<td>0.14 ± 0.00</td>
</tr>
<tr>
<td>pPProGreen (400mM)</td>
<td>63.10 ± 0.20</td>
<td>13.40 ± 0.40</td>
<td>17.87 ± 0.46</td>
<td>5.61 ± 0.14</td>
</tr>
<tr>
<td>pEYFP and pVLacGreen (1:1)</td>
<td>15.30 ± 1.08</td>
<td>49.13 ± 1.20</td>
<td>30.17 ± 2.30</td>
<td>5.41 ± 0.34</td>
</tr>
<tr>
<td>pEYFP/pVLacGreen (0mM)</td>
<td>51.87 ± 0.03</td>
<td>4.07 ± 0.11</td>
<td>42.80 ± 0.10</td>
<td>1.29 ± 0.04</td>
</tr>
<tr>
<td>pEYFP/pPProGreen (0mM)</td>
<td>3.39 ± 0.15</td>
<td>93.57 ± 0.15</td>
<td>0.30 ± 0.03</td>
<td>2.76 ± 0.03</td>
</tr>
<tr>
<td>pEYFP/pPProGreen (400mM)</td>
<td>11.37 ± 0.09</td>
<td>51.53 ± 0.15</td>
<td>0.80 ± 0.03</td>
<td>36.27 ± 0.22</td>
</tr>
</tbody>
</table>

When the GFP and YFP derivatives were co-expressed as was the case with DH5α(pEYFP/pPProGreen and pEYFP/pVLacGreen), the fluorescence intensity of each derivative was different than its fluorescence when present alone (Table 5). The fluorescence of YFP appeared to be slightly reduced in the presence of low concentrations of GFP in DH5α(pEYFP/pPProGreen) or DH5α(pEYFP/pVLacGreen) cells when compared to DH5α(pEYFP) cells. This may be due to YFP-GFP protein interactions reducing the quantum yield of YFP. In contrast, the fluorescence of YFP was increased when a moderate or high concentration of GFP was present. This result strongly suggests that fluorescent resonance energy transfer (FRET) occurs from GFP to YFP (Fig. 3). We hypothesize that FRET occurs more extensively at higher concentrations of proteins because of enhanced opportunities for protein-protein interactions. In this study, however, it
is not clear why at low GFP concentrations (i.e. in the strain with pEYFP/pVLacGreen), the GFP fluorescence was reduced so dramatically.

Figure 2. Representative histograms of the fluorescence from the lacZ-gfp expressing cells (green line) and lacZ-yfp expressing cells (yellow line) collected by a) the GFP detector and b) the YFP detector.

Table 5. The mean fluorescence of cells exhibiting GFP fluorescence (from quadrant 2 in Table 4) and cells exhibiting YFP fluorescence (from quadrant 3 in Table 4) in one representative study. Representative histograms of the fluorescence of the cells are found in Figure 2. Values represent the mean ± SEM for \( n = 3 \) samples.

<table>
<thead>
<tr>
<th>Strain</th>
<th>GFP (RFU) (^a)</th>
<th>YFP (RFU) (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pVLacGreen</td>
<td>18.47 ± 0.07</td>
<td>0.59 ± 0.00</td>
</tr>
<tr>
<td>pEYFP</td>
<td>ND(^c)</td>
<td>2.69 ± 0.00</td>
</tr>
<tr>
<td>pPProGreen (0mM)</td>
<td>37.67 ± 0.18</td>
<td>0.62 ± 0.02</td>
</tr>
<tr>
<td>pPProGreen (400mM)</td>
<td>285.50 ± 25.63</td>
<td>0.85 ± 0.05</td>
</tr>
<tr>
<td>pEYFP and pVLacGreen (1:1)</td>
<td>18.37 ± 0.09</td>
<td>2.25 ± 0.01</td>
</tr>
<tr>
<td>pEYFP/pVLacGreen</td>
<td>ND(^c)</td>
<td>0.62 ± 0.00</td>
</tr>
<tr>
<td>pEYFP/pPProGreen (0mM)</td>
<td>30.70 ± 0.06</td>
<td>0.98 ± 0.01</td>
</tr>
<tr>
<td>pEYFP/pPProGreen (400mM)</td>
<td>81.83 ± 1.59</td>
<td>5.97 ± 0.21</td>
</tr>
</tbody>
</table>

\(^a\) The data were generated using \( \lambda_{\text{ex}} = 488 \text{ nm} \) and \( \lambda_{\text{em}} = 510-520 \text{ nm} \).

\(^b\) The data were generated using \( \lambda_{\text{ex}} = 488 \text{ nm} \) and \( \lambda_{\text{em}} = 565-585 \text{ nm} \).

\(^c\) When percent of cells in a quadrant was less than 3%, the mean fluorescence was not meaningful and, therefore, was considered to be not detected (ND).
Figure 3. Proposed model of YFP-GFP interactions. All values represent the mean fluorescence of the indicated fluorescent protein. (○ = YFP, ■ = GFP)

**Sapphire**

The Sapphire derivative was also investigated as a second marker for GFP-expressing cells. It is excited by distinct wavelengths from GFP (optimum 399 nm), but exhibits a similar emission profile. Theoretically, distinct lasers could excite both GFP and Sapphire fluorescence and the emission could be collected by the same detector. The detection of fluorescence by the GFP-containing strain DH5α(pVLacGreen) after excitation with $\lambda_{\text{ex}} = 408$ nm indicates that this $\lambda_{\text{ex}}$ results in detectable GFP fluorescence, even after compensation (pVLacGreen, Table 6). Similarly, the detection of fluorescence by DH5α(pSapphire) after excitation with the $\lambda_{\text{ex}} = 488$ nm indicates that this wavelength results in detectable Sapphire emission, even after compensation (pSapphire, Table 6). The results
in detectable Sapphire emission, even after compensation (pSapphire, Table 6). The results
with all of the strains in Table 6 are consistent with this cross-over, demonstrating the
inadequacy of Sapphire as a second marker for GFP-marked cells. This was further
demonstrated by the reduced growth of the cells due to the presence of the Sapphire
derivative and elevated levels of GFP (data not shown). The unexpectedly high level of
fluorescence of DH5α(pSapphire/pPProGreen) in the presence of 200 mM NaCl could not
have occurred by FRET, since the two derivatives have the same emission profile.

Table 6. The mean fluorescence of strains using λex of 488 nm and λem of 525-535 for
detecting GFP, and λex of 408 nm and λem of 515-535 nm for detecting Sapphire. Values
represent the mean ± SEM for n = 2 samples.

<table>
<thead>
<tr>
<th>Strain</th>
<th>NaCl concentration (mM)</th>
<th>GFP (RFU)</th>
<th>Sapphire (RFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pVSP61</td>
<td>0</td>
<td>4.81 ± 0.01</td>
<td>3.94 ± 0.01</td>
</tr>
<tr>
<td>pVLacGreen</td>
<td>0</td>
<td>5.01 ± 0.06</td>
<td>4.03 ± 0.04</td>
</tr>
<tr>
<td>pSapphire</td>
<td>0</td>
<td>4.89 ± 0.02</td>
<td>4.17 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>4.86 ± 0.00</td>
<td>4.00 ± 0.01</td>
</tr>
<tr>
<td>pPProGreen</td>
<td>0</td>
<td>6.24 ± 0.61</td>
<td>4.70 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>30.60 ± 11.60</td>
<td>30.70 ± 11.50</td>
</tr>
<tr>
<td>pSapphire/pVLacGreen</td>
<td>0</td>
<td>6.05 ± 0.41</td>
<td>5.83 ± 0.50</td>
</tr>
<tr>
<td>pSapphire/pPProGreen</td>
<td>0</td>
<td>6.33 ± 0.03</td>
<td>5.85 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>51.15 ± 0.85</td>
<td>41.35 ± 0.35</td>
</tr>
</tbody>
</table>

Blue fluorescent protein

The BFP has very distinct excitation and emission spectra when compared to GFP
and therefore should be easily distinguished. Unfortunately, the 350 nm λ used to excite
BFP also excited GFP (pVLacGreen, Table 7), and the fluorescent yield of BFP was
exceptionally low (pEBFP, Table 7), as has been shown by others [e.g. (9, 10, 20)]. Due to
this overlap and the relatively low fluorescence of BFP, we were unable to distinguish BFP-
expressing cells from non-fluorescent cells (pEBFP vs. pVSP61, Table 7). Co-expression of
the lacZ-bfp fusion with the proU-gfp fusion significantly reduced the GFP fluorescence of
DH5α(pEBFP/pPProGreen) at various NaCl concentrations when compared to
DH5α(pPProGreen) at comparable concentrations (P<0.05 Student’s test) (Table 8).
Cultures of DH5α(pEBFP/pPProGreen) grew less at higher NaCl concentrations than
cultures producing a single fluorescent protein, suggesting that co-expression of BFP and
high levels of GFP was toxic to cells. The reduction of growth may account for the loss of
GFP fluorescence at the higher NaCl concentrations because cells may have had an
excessive metabolic load.
Table 7. Mean fluorescence of cells of DH5α containing various plasmids grown in ½-21C broth based on histograms. Values represent the mean ± SEM for n = 2 samples.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>GFP (RFU)</th>
<th>BFP (RFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pVSP61</td>
<td>ND</td>
<td>0.62 ± 0.03</td>
</tr>
<tr>
<td>pVLacGreen</td>
<td>5.64 ± 0.17</td>
<td>1.04 ± 0.17</td>
</tr>
<tr>
<td>pPProGreen</td>
<td>9.43 ± 0.19</td>
<td>0.99 ± 0.12</td>
</tr>
<tr>
<td>pEBFP</td>
<td>ND</td>
<td>0.42 ± 0.01</td>
</tr>
<tr>
<td>pEBFP/pVLacGreen</td>
<td>4.73 ± 0.52</td>
<td>0.46 ± 0.01</td>
</tr>
<tr>
<td>pEBFP/pPProGreen</td>
<td>7.00 ± 0.56</td>
<td>0.99 ± 0.02</td>
</tr>
</tbody>
</table>

* The data were generated using λ_{ex} = 488 nm and λ_{em} = 525-535 nm.
* The data were generated using λ_{ex} = 350 nm and λ_{em} = 440DL nm.
* When percent of cells in a quadrant was less than 3%, the mean fluorescence was not meaningful and, therefore, was considered to be not detected (ND).

Table 8. Osmoreponsiveness of proU-gfp fusion with and without the presence of BFP. Values represent the mean ± SEM for n = 2 samples.

<table>
<thead>
<tr>
<th>NaCl Concentration</th>
<th>pPProGreen GFP Mean Fluorescence (RFU)</th>
<th>Fold Induction</th>
<th>pEBFP/pPProGreen GFP Mean Fluorescence (RFU)</th>
<th>Fold Induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>0mM</td>
<td>9.43 ± 0.19</td>
<td>1.00 ± 0.02</td>
<td>7.00 ± 0.56</td>
<td>1.00 ± 0.08</td>
</tr>
<tr>
<td>50mM</td>
<td>18.85 ± 0.75</td>
<td>2.00 ± 0.08</td>
<td>15.45 ± 0.35</td>
<td>2.21 ± 0.05</td>
</tr>
<tr>
<td>100mM</td>
<td>36.85 ± 0.65</td>
<td>3.91 ± 0.07</td>
<td>26.00 ± 0.70</td>
<td>3.72 ± 0.10</td>
</tr>
<tr>
<td>200mM</td>
<td>76.40 ± 12.40</td>
<td>8.11 ± 1.32</td>
<td>42.30 ± 3.90</td>
<td>6.05 ± 0.56</td>
</tr>
<tr>
<td>400mM</td>
<td>161.30 ± 10.80</td>
<td>17.11 ± 1.15</td>
<td>21.10 ± 1.50</td>
<td>3.02 ± 0.21</td>
</tr>
</tbody>
</table>

DsRed

The DsRed protein is not a GFP derivative, and thus it may have less propensity to interact with GFP than the GFP derivatives examined. The use of a similar wavelength to excite both GFP and DsRed simplifies their analysis by flow cytometry. When examining DH5α(pVLacGreen) and DH5α(pPProGreen) by flow cytometry, it was clear that GFP fluorescence did not cross-over to the DsRed detector to any significant degree (Table 9). In contrast, when DsRed was produced in abundance, as in DH5α(pDsRed), the DsRed fluorescence did cross-over to the GFP detector (Table 9). When DsRed was produced in much lower quantities, as occurred in DH5α(pVLacRed), this cross-over was not detected (Table 9). The amount of fluorescence in the lacZ-DsRed-expressing strains was sufficient to distinguish them from non-fluorescent cells, which often exhibited fluorescence detected by the DsRed detector at a background level of approximately 4 (data not shown). When the GFP fluorescence of DH5α(pPLRProGreen), which expressed both lacZ-DsRed and proU-gfp, was compared to that of DH5α(pPProGreen) at various NaCl concentrations, the
GFP fluorescence of DH5α(pPLRProGreen) was consistently higher, with the magnitude of the difference being higher at elevated NaCl concentrations (Table 10). Thus, the presence of DsRed in the cell did increase GFP fluorescence in the presence of NaCl. During maturation, DsRed assumes a conformation that has spectral properties similar to those of GFP; this intermediate could account for the additional GFP fluorescence detected. If the GFP fluorescence of a cell is used as a quantitative indicator of promoter expression, as is true with the transcriptional fusion-based biosensor strain that we have constructed (1), then the quantitative contribution of DsRed to the GFP fluorescence would have to be accounted for when using a dual-marked strain.

Based on these positive results, we introduced pPLRProGreen into BRT98 and B728a and observed similar results to those of DH5α (Table 10). Following introduction of these strains onto bean plants, the DsRed fluorescence decreased dramatically and the introduced bacteria could not be distinguished from plant debris and non-fluorescent bacteria on the basis of DsRed fluorescence (data not shown). This loss of fluorescence may be due to the reduction in chromophore absorbency caused by the weak dimerization of the tetramers (2). Researchers have also reported that maturation of the protein can take hours to days and often incomplete maturation occurs (7). The loss of fluorescence may also be due to toxicity of the two proteins to the cells in planta; however, the presence of both DsRed and GFP in a cell did not alter the growth of B728a in culture even at 600 mM NaCl (Table 11). If toxicity in planta did occur, the construction of a fusion between a stronger promoter, P_piti, and an enhanced derivative of DsRed, DsRed2 (Clontech, Inc., Palo Alto, CA), which is generally less toxic to cells than DsRed (www.clontech.com), could overcome the detection problems encountered in planta. Our efforts thus far to construct this fusion, however, have not been successful.

Table 9. Mean fluorescence of strains in %-21C broth amended with 0 mM NaCl. Values represent the mean ± SEM for n = 3 samples.

<table>
<thead>
<tr>
<th>Strain ID</th>
<th>DH5α</th>
<th>BRT98</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GFP</td>
<td>DsRed</td>
</tr>
<tr>
<td>pVLacGreen</td>
<td>40.17 ± 3.30</td>
<td>0.51 ± 0.00</td>
</tr>
<tr>
<td>pPLRProGreen</td>
<td>11.13 ± 0.41</td>
<td>0.43 ± 0.00</td>
</tr>
<tr>
<td>pDsRed</td>
<td>8.41 ± 0.80</td>
<td>22.00 ± 0.10</td>
</tr>
<tr>
<td>pVLacRed</td>
<td>0.00 ± 0.00</td>
<td>5.37 ± 0.24</td>
</tr>
<tr>
<td>pPLRProGreen</td>
<td>12.50 ± 0.06</td>
<td>7.32 ± 0.12</td>
</tr>
</tbody>
</table>
Table 10. GFP fluorescence of proU-gfp-containing DH5α, BRT98 and B728a with and without the lacZ-DsRed fusion. Values represent the mean ± standard error of the mean for n = 3 samples.

<table>
<thead>
<tr>
<th>NaCl Conc</th>
<th>DH5α</th>
<th>BRT98</th>
<th>B728a</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM</td>
<td>pPProGreen</td>
<td>pPLRProGreen</td>
<td>pPProGreen</td>
</tr>
<tr>
<td></td>
<td>10.97 ± 0.26</td>
<td>12.50 ± 0.06</td>
<td>9.72 ± 0.08</td>
</tr>
<tr>
<td>200 mM</td>
<td>20.50 ± 1.85</td>
<td>45.97 ± 0.03</td>
<td>34.60 ± 1.85</td>
</tr>
<tr>
<td>400 mM</td>
<td>42.67 ± 2.76</td>
<td>106.33 ± 3.03</td>
<td>85.77 ± 2.13</td>
</tr>
<tr>
<td>600 mM</td>
<td>66.63 ± 0.94</td>
<td>129.20 ± 7.91</td>
<td>175.43 ± 24.96</td>
</tr>
</tbody>
</table>

Table 11. Optical density at 600 nm (OD_{600}) for the strain B728a in %-21C broth amended with NaCl after 48 h at 28°C. Values represent the mean ± standard error of the mean for n = 3 samples. Samples with an asterisk significantly differed from the corresponding sample of strains expressing only GFP fluorescence.

<table>
<thead>
<tr>
<th>NaCl Conc</th>
<th>pPProGreen</th>
<th>pPLRProGreen</th>
<th>pPNptGreen</th>
<th>pPLRNptGreen</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM</td>
<td>1.30 ± 0.02</td>
<td>1.24 ± 0.00</td>
<td>1.28 ± 0.00</td>
<td>1.28 ± 0.02</td>
</tr>
<tr>
<td>200 mM</td>
<td>0.73 ± 0.06</td>
<td>0.80 ± 0.02</td>
<td>0.83 ± 0.02</td>
<td>* 0.74 ± 0.02</td>
</tr>
<tr>
<td>400 mM</td>
<td>0.69 ± 0.17</td>
<td>0.55 ± 0.06</td>
<td>0.48 ± 0.16</td>
<td>0.44 ± 0.07</td>
</tr>
<tr>
<td>600 mM</td>
<td>0.03 ± 0.00</td>
<td>* 0.02 ± 0.00</td>
<td>0.02 ± 0.00</td>
<td>0.02 ± 0.00</td>
</tr>
</tbody>
</table>

PhoA

We introduced a constitutively expressed phoA gene into E. coli and evaluated the use of PhoA and a fluorescence substrate as a second marker in a proU-gfp-containing strain. The PhoA protein, alkaline phosphatase, cleaves the substrate MUP into a fluorescent product. The co-expression of GFP and PhoA in a cell did not reduce the size of the population that developed in %-21C medium in 48 h (data not shown); therefore, co-expression of GFP and PhoA was not toxic to the cells. However, PhoA-expressing cells could not be distinguished from non-fluorescent cells because the MUP-based fluorescence was quite low (pMS2B6, Table 12); in fact, it was lower than the small cross-over of the GFP fluorescence onto the MUP detector (pVLacGreen, Table 12). The fluorescence of pVLacGreen and pPProGreen cells with and without co-expression of phoA demonstrated that the presence of phoA reduced GFP fluorescence (Table 12). Furthermore, the presence of the phoA fusion significantly reduced proU-gfp expression at all salt concentrations (Tables 13).
Table 12. Mean fluorescence of E. coli Top10F' cells containing various fusions in 1/2-21C broth amended with 0 mM NaCl. Values represent the mean ± SEM for n = 2 samples.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>GFP</th>
<th>MUP</th>
</tr>
</thead>
<tbody>
<tr>
<td>pVSP61</td>
<td>0.43 ± 0.00</td>
<td>ND</td>
</tr>
<tr>
<td>pVLacGreen</td>
<td>6.32 ± 0.00</td>
<td>0.76 ± 0.09</td>
</tr>
<tr>
<td>pPProGreen</td>
<td>5.52 ± 0.08</td>
<td>0.53 ± 0.02</td>
</tr>
<tr>
<td>pMS2B6</td>
<td>ND</td>
<td>0.42 ± 0.02</td>
</tr>
<tr>
<td>pMS2B6/pVLacGreen</td>
<td>1.04 ± 0.00</td>
<td>0.46 ± 0.01</td>
</tr>
<tr>
<td>pMS2B6/pPProGreen</td>
<td>2.25 ± 0.24</td>
<td>0.42 ± 0.01</td>
</tr>
</tbody>
</table>

a The data were generated using λ_{ex} = 488 nm and λ_{em} = 525-535 nm.
b The data were generated using λ_{ex} = 350 nm (a UV blocker was used (< 350 nm)) and λ_{em} = 440DL nm.
c When percent of cells in a quadrant was less than 3%, the mean fluorescence was not meaningful and, therefore, was considered to be not detected (ND).

Table 13. GFP fluorescence of proU-gfp-containing E. coli Top10F' cells with and without the phoA fusion. Values represent the mean ± SEM for n = 2 samples.

<table>
<thead>
<tr>
<th>NaCl Conc (mM)</th>
<th>pPProGreen</th>
<th>pMS2B6/pPProGreen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MnX</td>
<td>Fold Induction</td>
</tr>
<tr>
<td>0mM</td>
<td>5.52 ± 0.08</td>
<td>1.00 ± 0.08</td>
</tr>
<tr>
<td>50mM</td>
<td>13.30 ± 0.05</td>
<td>2.41 ± 0.05</td>
</tr>
<tr>
<td>100mM</td>
<td>22.10 ± 0.24</td>
<td>4.01 ± 0.24</td>
</tr>
<tr>
<td>200mM</td>
<td>27.80 ± 0.16</td>
<td>5.04 ± 0.16</td>
</tr>
<tr>
<td>400mM</td>
<td>23.50 ± 0.45</td>
<td>4.26 ± 0.45</td>
</tr>
</tbody>
</table>

Syto red stain #64

We evaluated the effectiveness of the nucleic acid binding dye Syto Red dye #64 as a second fluorescent marker in proU-gfp-expressing cells. Based on its use in eukaryotes and some prokaryotes, this dye had the potential to mark cells without influencing cellular viability, and to be maintained through multiple generations (Molecular Probes, personal communication). Syto Red dye #64 was selected after finding that cells stained with the Syto Red dyes # 17, 59, 60, 61, 62 and 63 could not be distinguished from non-fluorescent cells throughout a 48 h staining period, whereas cells stained with Syto Red dye #64 could.

Populations of the 3 strains, DH5α, BRT98 and B728a, were non-uniformly stained with Syto Red dye #64 (Fig. 4 and Table 14). E. coli stain DH5α exhibited the most efficient uptake of the dye. Based on the percent of events in quadrants 3 and 4, approximately 72% of the DH5α cells were stained, whereas only 33 to 38% of the BRT98 and B728a populations were stained, respectively (Fig. 4 and Table 14). The presence of the dye significantly reduced GFP fluorescence of the proU-gfp expressing cells for all three strains (Table 15). Bacterial cell growth for all strains was severely reduced by the presence of the
dye when compared to cells without the dye. This was true even at the initial time point (Fig. 5). BRT98 and B728a cells stained with the dye recovered within 20 h and 30 h, respectively. The DH5α cells exhibited growth only after 35 h and by 48 h had not reached the population size of the culture without the dye.

---

**Figure 4.** Density plots of cells stained with Syto red dye #64. a) *E. coli* strain DH5α(pVLacGreen), b) *P. agglomerans* strain BRT98(pVLacGreen), and c) *Ps. syringae* strain B728a(pPNptGreen). Quadrant 1 contains cells or debris with no or low levels of fluorescence. Quadrant 2 contains cells exhibiting GFP fluorescence. Quadrant 3 contains cells exhibiting Syto fluorescence. Quadrant 4 contains cells exhibiting both GFP and Syto fluorescence. Values represent the mean ± SEM for *n* = 2 samples. The x- and y- axes represent GFP and Syto Red dye #64 fluorescence, respectively (range from 0.1 to 1000).

---

**Table 14.** Percent of population for each strain stained with Syto red dye #64 in the quadrants described in Figure 4. Values represent the mean ± standard error of the mean for *n* = 2 samples.

<table>
<thead>
<tr>
<th>Strain ID</th>
<th>Quadrant 1</th>
<th>Quadrant 2</th>
<th>Quadrant 3</th>
<th>Quadrant 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α pVLacGreen</td>
<td>25.85 ± 0.95</td>
<td>1.40 ± 0.00</td>
<td>25.80 ± 1.60</td>
<td>46.95 ± 0.65</td>
</tr>
<tr>
<td>BRT98 pVLacGreen</td>
<td>65.95 ± 8.05</td>
<td>0.60 ± 0.40</td>
<td>20.60 ± 3.00</td>
<td>12.85 ± 10.65</td>
</tr>
<tr>
<td>B728a pPNptGreen</td>
<td>54.55 ± 3.25</td>
<td>7.40 ± 1.00</td>
<td>18.20 ± 0.30</td>
<td>19.95 ± 2.55</td>
</tr>
</tbody>
</table>

**Table 15.** The mean GFP and Syto Red dye #64 fluorescence of three strains in ½-21C broth after 48 h. Values represent the mean ± SEM for *n* = 2 samples.

<table>
<thead>
<tr>
<th>Strain ID</th>
<th>Syto stain</th>
<th>GFP</th>
<th>Syto</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli pVLacGreen</td>
<td>-</td>
<td>4.70 ± 0.08</td>
<td>0.23 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>3.11 ± 0.10</td>
<td>1.33 ± 0.03</td>
</tr>
<tr>
<td>BRT98 pVLacGreen</td>
<td>-</td>
<td>7.52 ± 0.23</td>
<td>0.29 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>4.18 ± 1.01</td>
<td>0.39 ± 0.11</td>
</tr>
<tr>
<td>B728a pPNptGreen</td>
<td>-</td>
<td>1.80 ± 0.03</td>
<td>0.29 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.09 ± 0.01</td>
<td>0.39 ± 0.02</td>
</tr>
</tbody>
</table>

**PKH26 cell tracker**

We also examined the effectiveness of PKH26 cell tracker dye to stain bacterial cells. This dye binds to cell membranes and although it is reduced in half with each
subsequent generation, it remains detectable for ten generations in eukaryotic cells. Similar to the Syto Red stain #64, the cells were not uniformly labeled by the PKH26 stain; however, a higher percent of cells of each strain were stained when compared to the Syto stain (Fig. 4 and Table 16). After 3 min of incubation with PKH26, approximately 64%, 53% and 14% of the DH5α, BRT98, and B728a cells were stained, respectively. PKH26-stained cells were easily distinguished from non-fluorescent cells. The stain was lost as the populations grew, as expected (Fig. 5). For the *E. coli* strain, the stain was lost within 1 generation and for both *P. agglomerans* and *Ps. syringae* strains, the stain was lost within 10 generations (Fig. 5). The presence of the PKH26 stain reduced GFP fluorescence in DH5α and BRT98, but actually increased GFP fluorescence in B728a cells (Table 17).
Figure 5. Population sizes for *Escherichia coli* strain DH5α, *Pantoea agglomerans* strain BRT98 and *Pseudomonas syringae* pv. *syringae* strain B728a in 1/2-21C broth in the presence (●) and absence (○) of Syto Red dye #64 (0.01 mM/μl). Values represent the mean ± SEM for *n* = 2 samples.
Figure 6. Density plots of cells stained with PKH26 cell tracker dye. a) *E. coli* strain DH5α(pVLacGreen), b) *P. agglomerans* strain BRT98(pVLacGreen), and c) *Ps. syringae* strain B728a(pPNptGreen). Quadrant 1 contains cells or debris with no or low levels of fluorescence. Quadrant 2 contains cells exhibiting GFP fluorescence. Quadrant 3 contains cells exhibiting PKH26 fluorescence. Quadrant 4 contains cells exhibiting both GFP and PKH26 fluorescence. Values represent the mean ± SEM for *n* = 2 samples. The X- and Y-axes represent GFP and PKH26 fluorescence, respectively (range from 0.1 to 1000).

Table 16. Percent of population for each strain stained with PKH26 cell tracker dye in the quadrants described above.

<table>
<thead>
<tr>
<th>Strain ID</th>
<th>Quadrant 1</th>
<th>Quadrant 2</th>
<th>Quadrant 3</th>
<th>Quadrant 4</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> pVLacGreen</td>
<td>33.2</td>
<td>0.80</td>
<td>0.80</td>
<td>64.4</td>
</tr>
<tr>
<td>BRT98 pVLacGreen</td>
<td>43.7</td>
<td>0.39</td>
<td>0.39</td>
<td>52.8</td>
</tr>
<tr>
<td>B728a pPNptGreen</td>
<td>15.4</td>
<td>70.8</td>
<td>0.12</td>
<td>13.7</td>
</tr>
</tbody>
</table>

Table 17. The mean GFP and PKH26 fluorescence based on histograms of all three strains in ½-21C broth after 20 h. Values represent the mean ± standard error of the mean for *n* = 2 samples.

<table>
<thead>
<tr>
<th>Strain ID</th>
<th>PKH26 stain</th>
<th>GFP</th>
<th>PKH26</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> pVLacGreen</td>
<td>-</td>
<td>49.20 ± 1.10</td>
<td>1.09 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>24.45 ± 2.75</td>
<td>26.00 ± 1.10</td>
</tr>
<tr>
<td>BRT98 pVLacGreen</td>
<td>-</td>
<td>87.50 ± 2.50</td>
<td>1.76 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>49.25 ± 0.25</td>
<td>8.51 ± 3.30</td>
</tr>
<tr>
<td>B728a pPNptGreen</td>
<td>-</td>
<td>36.60 ± 0.40</td>
<td>1.05 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>40.90 ± 1.70</td>
<td>7.68 ± 0.46</td>
</tr>
</tbody>
</table>
Figure 7. PKH26 fluorescence (solid lines) and population sizes (dotted lines) for *Escherichia coli* strain DH5α(pVLacGreen), *Pantoea agglomerans* strain BRT98(pVLacGreen) and *Pseudomonas syringae* pv. *syringae* strain B728a(pPNptGreen) in the presence (●) and absence (○) of the PKH26 stain. Values represent the mean ± standard error of the mean for *n* = 2 samples.
Conclusions

We have attempted to identify a suitable second marker for our GFP-based reporter system. Unfortunately, we were unable to identify a marker that fulfilled all of our requirements for distinguishing our water-responsive bacterial biosensor from non-fluorescent bacteria or debris in planta while retaining GFP fluorescence as a quantitative measure of water availability. We investigated a variety of secondary markers including GFP derivatives, DsRed, PhoA, PKH26 and Syto Red dye #64, and characterized the strengths and limitations of each (Table 18). Our studies with the biosensor cells on plant leaves do not involve significant changes in population size of the biosensor cells, and are not weakened by the presence of unlabelled cells, since we will evaluate the GFP fluorescence of only those cells exhibiting the marker fluorescence. Thus far, the PKH26 stain shows the most promise for use in our studies; however, based on our results with DsRed, it is important that PKH26 stained cells be examined on the leaves. Furthermore, alternative staining conditions should be examined to evaluate if the proportion of cells in a population that are PKH26 stained can be enhanced. Ideally, we want a genetic system that can be easily transferred to a variety of strains to evaluate the water deprivation that bacteria sense on leaf surfaces. In the absence of a marker that could easily identify a variety of strains, strain-specific markers including antibodies and DNA probes can be developed.

Table 18. Limitations of the second markers tested

<table>
<thead>
<tr>
<th>Fluorescent marker</th>
<th>Toxic in culture</th>
<th>Sufficient fluorescence to distinguish from non-fluorescent cells</th>
<th>GFP fluorescence affected by 2nd marker</th>
<th>GFP excited by λ&lt;sub&gt;ex&lt;/sub&gt; used for 2nd marker</th>
<th>FRET occurred</th>
<th>Lost from cells during division</th>
<th>Population of cells were uniformly stained</th>
</tr>
</thead>
<tbody>
<tr>
<td>YFP</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>NA</td>
</tr>
<tr>
<td>Sapphire</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>BFP</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>DsRed</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>NA</td>
<td>No</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>MUP/PhoA</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Syto #64</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>NA</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>PKH26</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>NA</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

NA = not applicable

We report that FRET occurred between GFP and YFP within a cell when a high concentration of the proteins was present. Previous researchers have demonstrated FRET
between two fused proteins, with the return of fluorescence when the proteins dissociate. Several researchers have reported FRET either between BFP and GFP (10, 17, 18) or CFP and YFP (18). In all cases the proteins had been fused. Therefore, to our knowledge this is the first report that demonstrates energy transfer between GFP and YFP when the proteins are not fused. Generally, FRET is believed to occur when two proteins are within 100 angstroms of each other. If this is true, then the FRET that was observed at higher protein concentrations due to the induction of proU-gfp may have been caused by the crowding of the molecules.

In our experiments, many of the markers tested would not be suitable as a dual marker. For example, BFP, Sapphire and PhoA did not have sufficient fluorescence to distinguish the cells from non-fluorescing cells, and the BFP and Sapphire derivatives were toxic to the cells. Therefore, even in studies in culture, these three labels would not work to mark cells. The nucleic acid stain Syto Red #64 does label cells; however, the cells were no longer viable after staining. The YFP derivative could potentially be used depending on the application. For example, if the expression of the inducible gene was known to be sufficiently low, crowding and subsequent FRET would not occur. Also to avoid FRET, it may be possible to fuse a weak constitutive promoter to GFP and fuse the inducible promoter to YFP. Several characteristics of the markers tested may be advantageous for marking a strain with two fluorescent labels. For example, DsRed and GFP were successfully co-expressed without any detrimental effects on the cells, and were readily distinguished from each other. The limitation with DsRed is its interference with quantification of the GFP protein due to the apparent GFP-like fluorescence of the GFP-like intermediate. Even if the inducible promoter was fused to DsRed and the constitutive promoter fused to GFP, quantification still could not occur. Pairing DsRed with a different GFP derivative could overcome this problem. DsRed could work well with GFP in the same cell if both derivatives were under the control of constitutive promoters. Finally, the cell membrane dye, PKH26, successfully labeled several gram-negative bacteria, and could be useful in applications that do not involve extensive population growth since the dye is progressively lost from each subsequent generation.
Acknowledgements

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References


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