

Construction and Characterization of a *proU-gfp* Transcriptional Fusion That Measures Water Availability in a Microbial Habitat†

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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 (molecular weight, 8000). The fusion was induced to a detectable level by NaCl concentrations of 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.

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., references 43, 44, 47, and 48), 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 enterica* serovar 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) (49), fluorescence microscopy can be used to generate spatial information on 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 (41); 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

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fluorescence; thus, rapid induction events can be examined only if conditions that prevent further promoter activity during a postinduction incubation period can be imposed.

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 *E. coli* strain DH5 α (Gibco-BRL, Rockville, Md.), *P. syringae* pv. *Syringae* strain B728a (30), and *P. 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 Luria-Bertani medium (37) or in the low-osmotic medium one-half-strength 21C (1/2-21C) (16, 45). The 1/2-21C medium was supplemented with vitamin B₁ (0.0005%) for the growth of *E. coli* and was amended with NaCl, KCl, Na₂SO₄, or 8,000-molecular-weight polyethylene glycol (PEG 8000) to lower the water potential. The antibiotics rifampin and kanamycin were each used at a concentration of 50 μ g/ml. Cycloheximide (100 μ g/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, 49), 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, 50) was obtained from J. E. Loper.

To construct pVLacGreen, the *gfp* gene on pGreenTIR was amplified by using the primers 5'-CGAGCTCGAATTCT-3' and 5'-GAGCTCGAATTC-3', and a 752-bp *Eco*RI 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-3' and the downstream primer 5'-GCATACGTCGACATCCTGTCTCTTG-3'; the underlined bases indicate introduced *Sa*I sites. A 365-bp *Sa*I fragment containing the *nptII* promoter was inserted into pPROBE-KT, creating *P_{nptII-gfp}*. Plasmids were mobilized into strains DH5 α , BRT98, and B728a by triparental matings with the helper plasmid pRK2073 (6). The plasmid pPProGreen was constructed as described in Results.

Plant inoculation. The inocula for the plant studies were prepared by growing the strains on 1/2-21C plates for 48 h at 28°C. The cells were resuspended in 1/2-21C to a density of 10⁹ cells/ml for experiments involving bacterial recovery from leaves and flow cytometry and to a density of 10⁷ cells/ml for experiments involving microscopy of bacteria on leaves. Cell concentrations were determined turbidimetrically by using standard curves relating cell number to optical density at 600 nm 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 μ E m⁻² s⁻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 midvein, and each half was divided into thirds. The adaxial surfaces of three sections and the abaxial surfaces of three sections were examined with a Leica model DMIRB/E microscope equipped for epifluorescence. A filter set with an excitation wavelength of 450 to 490 nm and an emission wavelength of \geq 520 nm was used to visualize GFP production in *P. agglomerans* BRT98 cells on leaves. A filter set with an excitation wavelength of 340 to 380 nm and an emission wavelength of \geq 430 nm was used to visualize GFP production in *P. 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 with 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 (ThermoLynne-Barnstead, Dubuque, Iowa) with an excitation filter of 480 \pm 1 nm and an emission filter of \geq 515 nm and a FluoroMax2 (Jobin-Yvon-Horiba, Edison, N.J.), which was equipped with DataMax software (Jobin-Yvon-Horiba) and a 150-W xenon lamp. GFP fluorescence was measured with the FluoroMax2 using an excitation wavelength of 488 \pm 1 nm and an emission wavelength of 510 \pm 1 nm. Before analysis by fluorometry, the optical density at 600 nm of all cultures was adjusted to 0.05.

A Beckman-Coulter (Fullerton, Calif.) 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- \pm 20-nm band-pass filter. Voltages were set at 860 V for the GFP fluorescence detector, 790 V for side scatter, and 869 V 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, Fla.). Data analysis and graphical representation were done with FCS Express (version 1.0) software (De Novo 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 by 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 by 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 effects of distinct solutes on *P_{proU-gfp}* expression were compared by using 24-h broth cultures containing various NaCl, KCl, or Na₂SO₄ concentrations to confer water potentials of 0, -1, and -2 MPa. The concentrations of NaCl required to confer these water potentials were 0, 0.221, and 0.439 M, respectively; those of KCl were 0, 0.221, and 0.449 M, respectively; and those of Na₂SO₄ were 0, 0.171, 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 premoistened 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 (49). After the 2-h incubation period, three primary leaves were removed for each treatment and were each placed in 20 ml of 1/2-21C broth. The tubes were sonicated gently for 7 min to aid in bacterial recovery (40). The cells in a 50- μ 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- μ m pore size) followed by suspension in 3 ml of 1/2-21C. Samples were analyzed by 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 *Eco*RI-*Bam*HI fragment into the vector pPROBE-KT' upstream from the *gfp* gene, creating a *P_{proU-gfp}* transcriptional fusion; this plasmid was designated

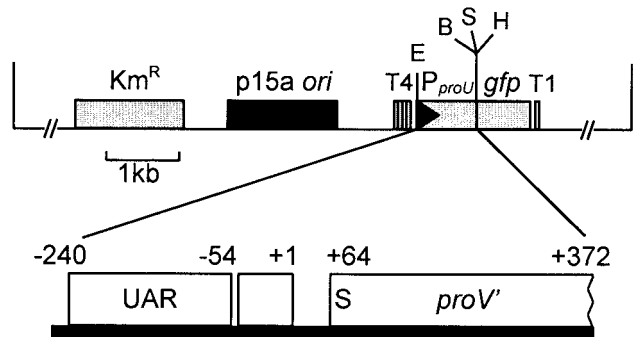


FIG. 1. Map of pPProGreen (15.2 kb). The promoter *proU* was directionally cloned from the *proU* expression vector pOSEX4 (19) into the broad-host-range pPROBE-KT' vector (38). The *rmB* transcriptional terminators, with one copy (T1) downstream and four copies (T4) upstream of the fusion, surrounded the *P_{proU}-gfp* fusion. 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. All of the restriction enzyme sites shown, including *Hind*III (H), *Sal*I (S), *Bam*HI (B), and *Eco*RI (E), were unique in pPProGreen.

pPProGreen. The *P_{proU}-gfp* fusion was flanked by T1 transcriptional terminators from the *E. coli* *rmB1* 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. 1). The requirement for these elements was confirmed in studies comparing the induction of *P_{proU}-gfp* fusions containing distinct regions of the *proU* promoter (data not shown).

Two additional plasmids were constructed to confer constitutive *gfp* expression in control strains. Plasmids containing *gfp* driven by the promoter of the lactose operon (*P_{lac}*) or the promoter of the neomycin phosphotransferase gene of *Tn5* (*P_{nptII}*) were constructed as described in Materials and Methods; these plasmids were designated pVLacGreen and pPNptGreen, respectively. Expression of *P_{nptII}-gfp* resulted in detectable fluorescence in all three strains, i.e., BRT98(pPNptGreen) (143 RFU), DH5 α (pPNptGreen) (65 RFU), and B728a(pPNptGreen) (34 RFU). In contrast, *P_{lac}-gfp* resulted in detectable fluorescence in BRT98(pVLacGreen) (524 RFU) and in DH5 α (pVLacGreen) (144 RFU) but not in B728a(pVLacGreen). *P_{lac}-gfp* expression appeared to be constitutive in both DH5 α (pVLacGreen) and BRT98(pVLacGreen) based on the absence of detectable induction by IPTG (isopropyl- β -D-thiogalactopyranoside) (data not shown). One possible reason for this may be because of the titration of the LacI repressor due to the presence of multiple (six to eight) copies of the plasmid (22).

Osmoinduction of *P_{proU}-gfp* in broth-grown *E. coli* cells. We characterized the effect of NaCl concentration on *P_{proU}-gfp* expression in DH5 α (pPProGreen) in broth-grown cells. Previous studies of *P_{proU}* expression in *S. enterica* serovar Typhimurium examined induction by a single NaCl concentration (13, 19, 32) or by multiple concentrations (14, 19), with the latter demonstrating that *P_{proU}* expression varies with NaCl

concentration. We observed a consistent increase in *P_{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. 2A). The *proU* promoter in DH5 α (pPProGreen) was not induced by NaCl concentrations greater than 400 mM (Fig. 2A), as was observed previously in *S. enterica* serovar Typhimurium (14). DH5 α (pPProGreen) did not grow at salt concentrations of greater than 500 mM NaCl.

A time course study using fluorometry demonstrated that induction of *P_{proU}-gfp* in DH5 α (pPProGreen) occurred rapidly. In previous studies with *E. coli* and *S. enterica* serovar Typhimurium, *P_{proU}* induction occurred within 10 min following exposure to NaCl (14, 33). We observed a detectable increase in *P_{proU}-gfp* expression in DH5 α (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 *P. syringae* cells. BRT98(pPProGreen) and DH5 α (pPProGreen) were similar in their basal levels of *P_{proU}-gfp* transcription in 1/2-21C broth. At NaCl concentrations of 200 mM or greater, *P_{proU}-gfp* was induced more in BRT98(pPProGreen) than in DH5 α (pPProGreen) (Fig. 2A and B). Similar to DH5 α (pPProGreen), BRT98(pPProGreen) exhibited a threshold NaCl concentration above which it did not exhibit further increases in fluorescence (Fig. 2B), and it was inhibited in growth by NaCl concentrations of greater than 500 mM.

B728a(pPProGreen) exhibited a significantly lower basal level of *P_{proU}-gfp* transcription in 1/2-21C broth (0 mM NaCl) than did either DH5 α (pPProGreen) or BRT98(pPProGreen) ($P < 0.05$). For example, in one study, the fluorescence of B728a(pPProGreen) in 1/2-21C broth was 3.3 ± 0.3 RFU, whereas those of DH5 α (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. 2C), was significantly less than that in DH5 α (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 those in the other strains at these NaCl concentrations (Fig. 2A to C). Similar to DH5 α (pPProGreen) and BRT98(pPProGreen), B728a(pPProGreen) could not grow in 1/2-21C broth with NaCl concentrations of greater than 500 mM.

The fluorescence of the strains DH5 α (pVLacGreen), BRT98(pVLacGreen), and B728a(pPNptGreen) was not affected by the NaCl concentration in the medium (Fig. 2A to 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 effects of NaCl concentration on *P_{proU}* expression in planktonic (broth-grown) cells (Fig. 2A to C) versus surface-grown (plate-grown) cells (Fig. 2D to F). The basal level of *P_{proU}-gfp* expression in cells grown on 1/2-21C plates was similar to that in cells grown in 1/2-21C broth (data not shown). In DH5 α (pPProGreen) cells, *P_{proU}* was induced more by NaCl concentrations of up to 400 mM in cells from plates than in

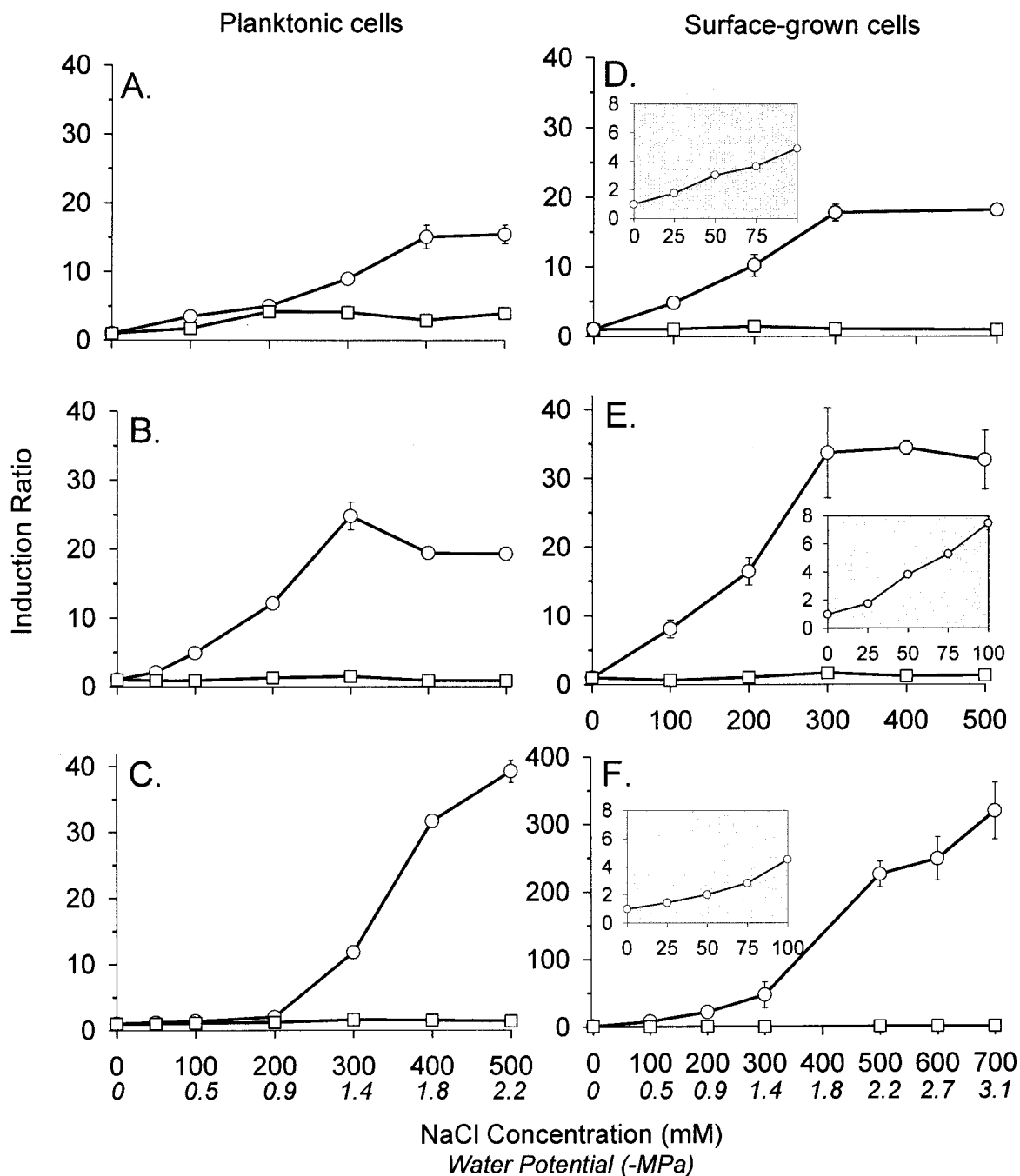


FIG. 2. Induction of $P_{proU-gfp}$ in cells that were grown in 1/2-21C broth (A, B, and C) or on 1/2-21C plates (D, E, and F) containing various concentrations of NaCl. (A and D) *E. coli* DH5 α (pPPProGreen) (○) and DH5 α (pVLacGreen) (□); (B and E) *P. agglomerans* BRT98(pPPProGreen) (○) and BRT98(pVLacGreen) (□); (C and F) *P. syringae* B728a(pPPProGreen) (○) and B728a(pPNptGreen) (□). The insets 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 \pm standard error of the mean ($n = 3$).

cells from broth (Fig. 2A and D). Similarly, P_{proU} was induced more in BRT98(pPPProGreen) and in B728a(pPPProGreen) in cells from plates than in cells from broth at comparable NaCl concentrations. In contrast to the two enteric species, BRT98(pPPProGreen) and DH5 α (pPPProGreen), the strain B728a(pPPProGreen) exhibited a dramatic increase in fluores-

cence as the NaCl concentration was increased from 300 to 700 mM in the plates (Fig. 2D to F), and B728a(pPPProGreen) grew on plates at NaCl concentrations of greater than 500 mM.

To identify the lower limit of detection by the biosensors, we exposed BRT98(pPPProGreen), DH5 α (pPPProGreen), and B728a(pPPProGreen) to NaCl concentrations of between 0 and

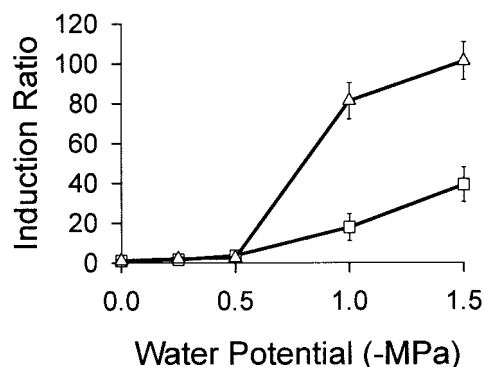


FIG. 3. Induction of $P_{proU-gfp}$ in cells that were grown on 1/2-21C plates containing various levels of PEG 8000. *P. agglomerans* BRT98(pPProGreen) (□) and *P. syringae* B728a(pPProGreen) (△) were grown for 24 h, and the fluorescence intensity of cell suspensions was measured with a fluorometer. The induction ratio was the mean fluorescence of the cells relative to the mean fluorescence of cells grown with 0% PEG 8000. Values represent the mean \pm standard error of the mean ($n = 3$).

100 mM. $P_{proU-gfp}$ expression in all three species detectably increased over the range from 25 to 100 mM NaCl (Fig. 2D to F, insets). In an additional experiment, the minimum NaCl concentration that significantly increased cellular fluorescence in DH5 α (pPProGreen) and B728a(pPProGreen) was 10 mM, and that in BRT98(pPProGreen) was 15 mM ($P < 0.05$) (data not shown).

$P_{proU-gfp}$ induction by a diversity of solutes. In addition to NaCl, the solutes KCl and Na₂SO₄ induced $P_{proU-gfp}$ expression in all three bacterial species over a range of water potentials (data not shown). For example, DH5 α (pPProGreen) and BRT98(pPProGreen) cells were induced two- to threefold by NaCl, KCl, and Na₂SO₄ levels conferring a water potential of -1 MPa. $P_{proU-gfp}$ thus responded to high external osmolarity rather than to ion toxicity. The *proU* promoter similarly responded to high external osmolarity in *S. enterica* serovar Typhimurium (14). The strains BRT98(pPProGreen), DH5 α (pPProGreen), and B728a(pPProGreen) were also grown on plates with the nonpermeating solute PEG 8000 (46), 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 (see, e.g., references 2, 10, and 16) and is thought to better simulate conditions in low-water-content habitats than do permeating solutes. Although DH5 α (pPProGreen) did not grow on 1/2-21C plates with PEG 8000, BRT98(pPProGreen) and B728a(pPProGreen) exhibited increasing $P_{proU-gfp}$ expression with increasing PEG 8000 concentrations (Fig. 3). 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. 2 and 3); 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. 2E and 3). In B728a(pPProGreen), $P_{proU-gfp}$ was induced approximately 48- and 100-fold by -1.5 -MPa conditions imposed by NaCl and PEG 8000, respectively (Fig. 2F and 3).

Spatial distribution of induced biosensor cells on bean leaf

surfaces. The spatial distribution of *P. agglomerans* and *P. syringae* cells exhibiting constitutive *gfp* expression was evaluated to identify the leaf surface regions that were colonized by these species under both high- 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 bases 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. 4A). 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 edges of the rings (Fig. 4B). 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.

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 premoistened tents to prevent further drying and to allow for GFP maturation to a fluorescent state (49). 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. 5A). 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

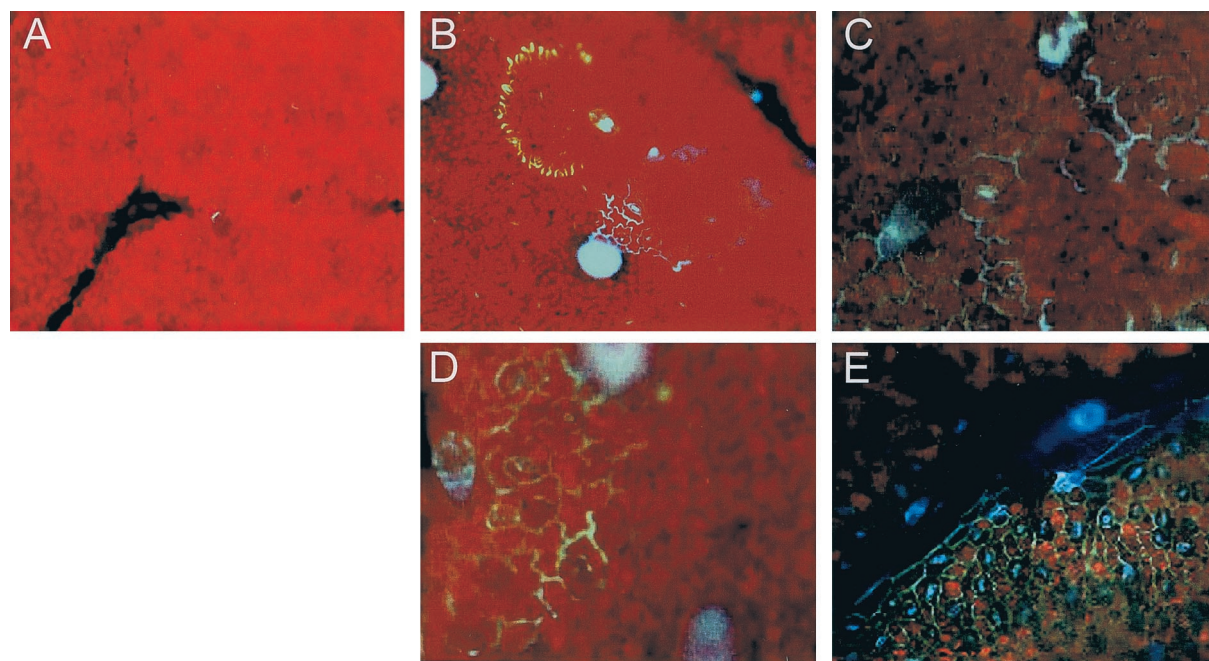


FIG. 4. Spatial distribution of cells exhibiting fluorescence due to $P_{proU-gfp}$ induction on bean leaves (*P. vulgaris*). (A) *P. agglomerans* BRT98(pPPProGreen) on a bean leaf after 24 h of incubation under moist conditions (no visible bacterial fluorescence); (B) *P. agglomerans* BRT98(pPPProGreen) on a bean leaf 4 h after transfer of the plants from moist conditions to ambient conditions (i.e., after 4 h of drying); (C) *P. syringae* B728a(pPPProGreen) on a bean leaf after 4 h of drying; (D) *P. agglomerans* BRT98(pPPProGreen) on a bean leaf after 8 h of drying; (E) *P. syringae* B728a(pPPProGreen) on a bean leaf after 8 h of drying. Leaves were examined by epifluorescence microscopy. The bacterial cells exhibited green or bluish-green fluorescence, the plant epidermal cells exhibited red autofluorescence, the leaf trichomes exhibited bluish-green (B to D) or blue (E) autofluorescence, and the stomata exhibited blue autofluorescence (E). Magnification, $\times 100$.

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 that at 0 min (Fig. 5A). 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 $x = \text{NaCl concentration}$ and $y = \text{ratio of the fluorescence at a given NaCl concentration to that at 0 mM}$), 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), and 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 Int. Symp. Microbiol. Aerial Plant Surfaces, abstr. 28, 2000; C. Riffaud and C. E. Morris, Abstr. 7th Int. Symp. Microbiol. 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 in experiment 1, there was relatively little leaf-to-leaf variation at any given time point, at least within the first hour (Fig. 5B). 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 in experiment 1, the mean fluorescence of the population significantly increased immediately following introduction of the bacterial cells onto 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(pPPProGreen) cells recovered at 35 and 60 min had increased by 1.61- and 1.67-fold relative to that at 0 min (Fig. 5B), 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(pPPProGreen) and BRT98(pVLacGreen) populations decreased significantly over time (for both strains, slope = -0.02 [$P < 0.01$]), suggesting that the cells decreased in size.

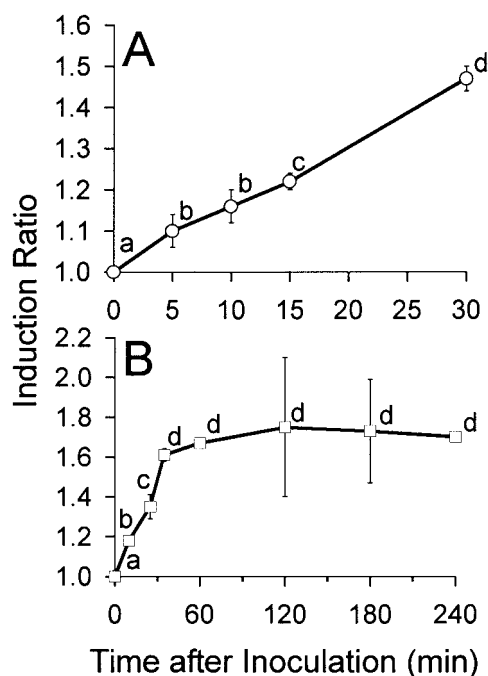


FIG. 5. Induction of $P_{proU}\text{-}gfp$ in *P. agglomerans* BRT98(pPPro-Green) cells recovered from bean leaves at various times following inoculation. (A) Experiment 1, in which the cells were monitored for 30 min ($n = 3$); (B) experiment 2, in which the cells were monitored for 240 min ($n = 2$). 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 \pm standard error of the mean for n samples. Data points indicated by the same letter do not differ by Fisher's least-significant difference test ($P < 0.05$).

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 ($P < 0.05$).

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 $P_{proU}\text{-}gfp$ fusion on a multicopy, 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, in both the absence and presence of high osmolarity, ensures its maintenance under conditions without antibiotic selection (data not shown). Finally, 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 $P_{proU}\text{-}gfp$ responded to water deprivation in *E. coli*, *P. agglomerans*, and *P. syringae*, as well as in *Xanthomonas campestris* pv. *Vesicatoria* (unpublished data).

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 of from 0 to 300 mM correlated with both an increase in the amount of GFP protein present, as detected by Western blotting (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 of 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. enterica* serovar Typhimurium. The basal level of P_{proU} expression in *P. syringae* was lower than that in *E. coli*, but the levels were 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 *P. syringae*. The osmoreponsiveness of the $proU$ promoter in *P. syringae* provides support for the hypothesis that $proU$ osmoregulation does not require specific regulatory proteins, although it is possible that *P. 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 (42). In fact, this difference has been demonstrated to influence bacterial survival following introduction onto plant leaves (51). In this study, the similarity in the levels of $P_{proU}\text{-}gfp$ 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 they are likely to be more similar physiologically 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* DH5 α was of less interest because it is not a normal inhabitant of leaf surfaces, and *P. 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 of 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 rates 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 that 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 to 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 to cause cell death or perhaps even to reduce growth. If we assume that bacteria and solutes are uniformly distributed throughout a water layer on a leaf and 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 to 35 μ m.

Macroscopic 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, 11, 23, 25, 36). 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 $P_{proU-gfp}$ 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 of 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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