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Diverse Microhabitats Experienced by *Halomonas variabilis* on Salt-Secreting Leaves

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The leaf surfaces of the salt-excreting tree *Tamarix aphylla* harbor a wide diversity of halophilic microorganisms, including *Halomonas* sp., but little is known of the factors that shape community composition in this extreme habitat. We isolated a strain of *Halomonas variabilis* from the leaf surface of *T. aphylla* and used it to determine the heterogeneity of salt concentrations experienced by bacteria in this environment. This halophilic strain was transformed with a *proU::gfp* reporter gene fusion, the fluorescence of which was responsive to NaCl concentrations up to 200 g liter^{-1}. These bioreporting cells were applied to *T. aphylla* leaves and were subsequently recovered from dew droplets adhering to the leaf surface. Although cells from within a given dew droplet exhibited similar green fluorescent protein fluorescence, the fluorescence intensity varied between droplets and was correlated with the salt concentration measured in each drop. Growth of *H. variabilis* was observed in all droplets, regardless of the salt concentration. However, cells found in desiccated microniches between dew drops were low in abundance and generally dead. Other bacteria recovered from *T. aphylla* displayed higher desiccation tolerance than *H. variabilis*, both in culture and on inoculated plants, despite having lower osmotic tolerance. Thus, the *Tamarix* leaf surface can be described as a salty desert with occasional oases where water droplets form under humid conditions. While halotolerant bacteria such as *Halomonas* grow in high concentrations of salt in such wet microniches, other organisms are better suited to survive desiccation in sites that are not wetted.

The leaf surfaces of *Tamarix aphylla* (hereafter referred to as *Tamarix*), a resilient tree capable of growth in soils having a wide variety of water availabilities, are colonized by a diverse microbial community (1, 2). *Tamarix* is successful in saline soils due to an adaptation mechanism that allows it to excrete excess salt onto its scale-like leaf surface. Some of the salt is subsequently shed from the leaf and salinizes the topsoil, thereby inhibiting the growth of other plants and thus enabling some members of this genus to be aggressive invasive species (3). The high levels of salt (NaCl and other salts) and other exudates on the leaves are hygroscopic, and liquid water readily forms droplets on the leaves when the ambient humidity is sufficiently high (4). Chemical characterization of dew droplets and leaf washes from *Tamarix* reveal that the leaf surface has a high pH (pH $>$ 9.0) and dissolved salt concentrations that are at least five times higher than those of seawater (2). Thus, in addition to dealing with the normally harsh conditions encountered on all leaves such as high UV irradiation flux and fluctuating temperatures (5), *Tamarix* epiphytes must also contend with high pH and salinity.

Prior research found that the leaves of *Tamarix* are mainly colonized by halophilic microorganisms adapted to this highly saline environment (2). Although trees at all sites sampled harbored a high proportion of halophilic bacteria, the species compositions of these communities varied dramatically at different geographical sites. For instance, *Halomonas* was the dominant genus (ca. 90% of total 16S reads) on *Tamarix* samples collected near the Mediterranean Sea, but was a minor component of communities on trees near the Dead Sea (1). This disparity was hypothesized to reflect differences in local selective factors such as the higher salinity, temperature or lower air humidity encountered near the Dead Sea, although this was not directly tested. In order to understand the factors that contribute to the successful colonization of bacteria in this unique habitat, it is necessary to characterize the nature of the immediate surroundings of the bacteria on the leaf surface, as well as their responses to such conditions. For this purpose, we utilized a *Halomonas variabilis* strain recovered from *Tamarix* as a model organism to interrogate the leaf surface since the prevalence of this genus on the leaves of many *Tamarix* trees suggests that it is well adapted to life on a salty plant. Members of the genus *Halomonas* are commonly isolated from salt lakes and brines, saline soils, and salted food products and display some of the most extensive salt tolerance observed in prokaryotes (6). These moderate halophiles are gaining considerable attention for their potential use in biotechnological applications because of their ease of culture and amenability to genetic manipulation (7, 8). Although there have been many culture-based investigations into the physiology of these organisms (9), detailed studies of *in situ* colonization by *Halomonas* species are lacking (6). Observations of the epiphytic growth of *H. variabilis* on *Tamarix* should provide insight into the factors that determine the occurrence of *Halomonas* in different *Tamarix* communities, as well as expand our general knowledge of this important genus which has great biotechnological promise.

Whole-cell bioreporters are useful in assessing the spatial variability of resources in a habitat. For instance, while chemical analysis of leaf washes can reveal the leaf-to-leaf variability of resources such as sugars (10), any microscale variation of such resources that greatly affect the success of bacterial colonization (11) cannot be ascertained by such measurements. Similarly, although analyses of dew or leaf washes can reveal overall salinity levels, individ-

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ual bacteria might perceive a broad range of local salinities on leaf surfaces. Thus, bioreporters based on salt-responsive promoters could reveal microscale heterogeneity. The *proU* operon encodes an ABC transporter of the compatible solute glycine betaine and is induced with increasing osmolarity (by NaCl and other salts) (12). The regulatory mechanisms of this promoter are well characterized and include at least three independent regulatory elements (12) that appear to be well conserved among many microorganisms. Bioreporter strains such as *Pantoea agglomerans* and *Pseudomonas syringae* harboring an *E. coli proU* promoter fused to a *gfp* reporter gene have previously provided considerable information about water availability on the leaves of plants such as bean and *Arabidopsis*, although the reporter species could only tolerate salinity up to about 30 g of NaCl liter^{-1} (13–15). However, dissolved salts can reach 220 g liter^{-1} on wetted *Tamarix* leaves (2), which would prohibit the use of such bioreporter strains for investigation of phyllosphere microhabitats on this species. A halophilic strain harboring a salinity reporter construct would thus expand the range of salinity detectable in biological settings. In this report, we characterize the halotolerance of a *Halomonas* strain from *Tamarix* and demonstrate its utility as an *in situ* salinity bioreporter strain. Furthermore, we compare this strain to other taxa from *Tamarix* in order to ascertain its limitations as a bioreporter in this setting, as well as to address previous observations of its distribution on natural stands of *Tamarix* trees. Measurements of its activity and viability in different microsites on leaves reveal that the *Halomonas* strain tolerates locally high salinity levels but not the absence of water, a lifestyle that is distinct from other *Tamarix* epiphytes that exhibit a much higher tolerance of desiccation but a lower salt tolerance.

MATERIALS AND METHODS

Bacterial isolates and growth conditions. *T. aphylla* leaves were collected from a site on the University of California Campus in Davis, CA, in late September 2010. Samples were immersed in phosphate washing buffer (10 mM, pH 7.5) and sonicated for 2 min; appropriate dilutions were plated on *Tamarix* medium (TXM; 3.4 g of pancreatic casein digest liter^{-1}, 0.6 g of papaic soybean digest liter^{-1}, 0.5 g of dextrose liter^{-1}, 0.5 g of K_2HPO_4 liter^{-1}, 30 g of NaCl liter^{-1}) containing 1.5% agar. Strains were grown at 28°C. Bacteria used in growth and fluorescence response curves were first precultured in the designated media for 24 h before diluting the culture and monitoring growth or fluorescence. Antibiotics were used at the following concentrations: kanamycin, 50 μg ml^{-1}, and natamycin, 21.6 μg ml^{-1}.

Bacterial identification. Genes encoding 16S rRNA were amplified by colony PCR using the universal primers 27F (5′-AGAGTTTGATCCTGG CTCAG) and 1492R (5′-TACGGYTACCTTGTTACGACTT) (16). PCR conditions were as follows: an initial denaturation of 10 min at 95°C, 28 cycles of 95, 59, and 72°C for 1 min each, with a final extension time of 10 min at 72°C. Products were excised from an agarose gel, extracted (UltraClean GelSpin; MoBio, CA), and submitted for sequencing at the UC Berkeley Sequencing Facility. The 16S rRNA gene sequences were compared to GenBank databases using BLAST (http://www.ncbi.nlm.nih.gov).

Plant inoculations. *T. aphylla* cuttings were taken from the same trees in Davis, CA, that were used for bacterial isolations. Cuttings were rooted in conical pots with soil composed of 10 parts vermiculite, 3 parts soil, 2 parts peat moss, and 1 part sand. Plants were grown with bottom-watering for at least 6 months in a greenhouse. The secreted salt levels on some plants were increased by two additions of 1 liter of 0.2 M NaCl to the watering tray over a 1-month period prior to experimentation. Bacterial inoculum was cultured on TXM plates, suspended and washed in sterile potassium phosphate buffer (10 mM [pH 7.5]) and applied to plants at a final cell concentration of 10^6 cells ml^{-1}. Cell suspensions were applied as a fine mist onto plants using a hand-held perfume mister, and the plants were then enclosed in a transparent plastic chamber with open trays of water to maintain 95 to 100% humidity and incubated at 25°C with a 16-h light period each day for 2 days. The plant chamber was then opened, and the plants were allowed to dry at ambient room conditions (ca. 50% humidity) for 2 days before sampling.

Dissolved salts. Individual droplets that had formed on leaves were removed by aspiration, their volume measured, and brought to 1-ml final volume with double-distilled H_2O. The diluted samples were filter sterilized and electrical conductivity (EC) was measured using a conductivity meter (S30 SevenEasy Conductivity; Mettler, OH). The relationship between EC values (mS cm^{-1}) and the concentration of Na^+ ions in the leaf wash (mg liter^{-1}) was established using an inductively coupled plasma optic emission spectrometer (ICP/OES; Optima 3000; Perkin-Elmer, MA).

Desiccation assay. Desiccation assays were performed similarly to Stockwell et al. (17). Cells grown on TXM plates were suspended in TXM to an optical density at 600 nm (OD_{600}) of 0.1- and 10-μl aliquots were applied to 1-cm^2 discs of sterile filter paper (#3; Whatman, Kent, United Kingdom). Paper discs were suspended for various times in chambers containing saturated NaCl solutions in order to maintain a constant 75% humidity at 25°C. The samples were then immersed in 1 ml of TXM, sonicated, vortexed, and appropriate dilutions applied to TXM plates to determine the number of surviving CFU.

Generation of a kanamycin-resistant *Psychrobacter*. *Psychrobacter alimentarius* strain Tx7, recovered from the leaves of *T. aphylla*, and the *E. coli* conjugal donor strain BW20767 harboring plasmid pRL27 (18) containing a kanamycin resistance-conferring transposon were grown overnight on agar plates. Cells were then resuspended in phosphate buffer and mixed at a ratio of 1:3 (*E. coli* to *Psychrobacter*) and incubated overnight as a confluent lawn on a King's medium B plate. After incubation, the cells were resuspended in phosphate buffer, plated onto TXM medium containing kanamycin, and grown at 4°C. The identities of kanamycin-resistant *Psychrobacter* transconjugants were confirmed by 16S rRNA sequencing.

GFP fluorescence. *H. variabilis* was transformed by electroporation with either plasmid p519*n-gfp* conferring constitutive green fluorescent protein (GFP) fluorescence (19) or plasmid pPProGreen conferring osmotically inducible GFP fluorescence (13). Strains were grown on TXM plates for 2 days and were either inoculated onto plants as described above or suspended in phosphate buffer and added to modified TXM medium containing various NaCl concentrations. The GFP fluorescence intensity (excitation/emission wavelength of 485 nm/528 nm) and the OD_{600} values were determined for culture-grown cells using a Synergy 2 multimode microplate reader (BioTek). A relative fluorescence unit was defined as the fluorescence of a suspension normalized for its turbidity measured as OD_{600}.

Quantitative microscopy. Inoculated leaves were harvested and sonicated in 30 ml of phosphate buffer for 2 min. The cells were collected by centrifugation and flash-frozen in a solution containing 2 μg of DAPI (4′,6′-diamidino-2-phenylindole) ml^{-1}. Alternatively, for propidium iodide (PI) staining, harvested cells were incubated for 10 min at room temperature in a solution containing 2 μg of PI ml^{-1}, followed by a wash step to remove excess PI prior to fixation. Stained cells were fixed in 4% paraformaldehyde for 15 min, washed three times in phosphate buffer, air dried onto charged slides, and mounted in Aqua PolyMount (Polysciences). Bacteria were photographed at ×1,000 magnification using a Zeiss AxioImager M1 microscope equipped with a Hamamatsu digital camera. Samples were excited using a broad-spectrum mercury arc lamp and visualized using standard DAPI, rhodamine, and EndowGFP filter cubes. For each treatment, 5 to 10 images were acquired using iVision software using the Multi-D Acquire function for paired photomicrographs. For all image pairs, DAPI-stained bacterial cells were masked using the iVision Segmentation function, and the segment masks were

pasted onto the tandem GFP images. Mean GFP pixel intensity for each masked object was quantified and background fluorescence was subtracted.

Confocal microscopy. Inoculated leaves were incubated in a humid chamber for 2 days and then immediately sampled. *Tamarix* leaves were fixed in 4% paraformaldehyde solution for 15 min, gently washed in phosphate buffer, hand sectioned, and mounted. Epiphytic bacteria were imaged at ×1,000 magnification using a Zeiss LSM 710 confocal microscope. Fluorescent *H. variabilis* cells were visualized using an argon laser (488 nm, 4% intensity). Images of the bacterial communities on nonplanar *Tamarix* leaves were obtained by compressing z-stack GFP images into a two-dimensional maximum intensity z-stack projection via ImageJ analysis software (v1.44). A differential interference contrast image of a given field of view was overlaid on the compressed GFP image of the z-stack image to provide a sense of the spatial location of *H. variabilis* on *Tamarix*.

Statistical analysis. Most data and regression analysis was carried out using Statistica (StatSoft, Tulsa, OK). Simple linear regressions were tested for significance by determining whether or not the slope was significantly different from zero.

Nucleotide sequence accession numbers. Sequence data have been submitted to the GenBank database under accession numbers JX034739 to JX034741.

RESULTS

NaCl tolerance of *H. variabilis* strain Tx42. A variety of salt-tolerant bacteria were isolated from *Tamarix* leaves from trees grown in central California and identified by 16S rRNA sequencing. A strain that shared 99% 16S sequence identity with *Halomonas variabilis*, designated as Tx42, was selected for more detailed examination. Growth of *H. variabilis* strain Tx42 was observed in broth cultures having a range of salinities from 15 to 200 g of NaCl liter^{-1} at 28°C. Because growth lags were observed for cells upon initial exposure to higher salinity concentrations, cells were precultured for 24 h in respective media salinities prior to starting any salinity-dependent experiments. Optimal growth with a doubling time of about 1.5 h was observed in medium containing 30 g of NaCl liter^{-1} (Fig. 1A). The division of *H. variabilis* slowed at higher salinity levels, although growth was observed up to 200 g of NaCl liter^{-1}. Similar to other halophiles (6), the NaCl tolerance of *H. variabilis* varied with culture conditions; lowering the incubation temperature to 24°C enabled growth in broth culture at salinities as low as 5 g of NaCl liter^{-1}. Decreasing the shaker incubation speed or increasing the initial inoculum density (from ~0.015 to ~0.15) also enabled cell division of *H. variabilis* at lower salinities as indicated by increased optical density and successful passage (data not shown). Growth at 28°C was observed on solid media over the range of 2.5 to 150 g of NaCl liter^{-1}, similar to that reported for other *Halomonas* strains (9). In addition, growth was observed up to 41°C, which is above the average maximum temperature bacteria would have been expected to encounter in the arid desert samples (1, 20). This indicates that the low relative *Halomonas* populations observed in arid desert samples were likely caused by factors other than temperature.

Epiphytic colonization of *H. variabilis* on *Tamarix* leaves. Because *Halomonas* strains were frequently the dominant genus encountered on *Tamarix* trees in various Mediterranean and U.S. sites (1, 2), it was expected that *H. variabilis* would successfully colonize greenhouse-grown *Tamarix* plants. A plasmid conferring both kanamycin resistance and GFP fluorescence (p519*n-gfp*) was introduced into this strain to enable its specific recovery and to visualize its presence on leaves. The presence of *H. variabilis* (p519*n-gfp*) on *Tamarix* leaves was determined by monitoring its

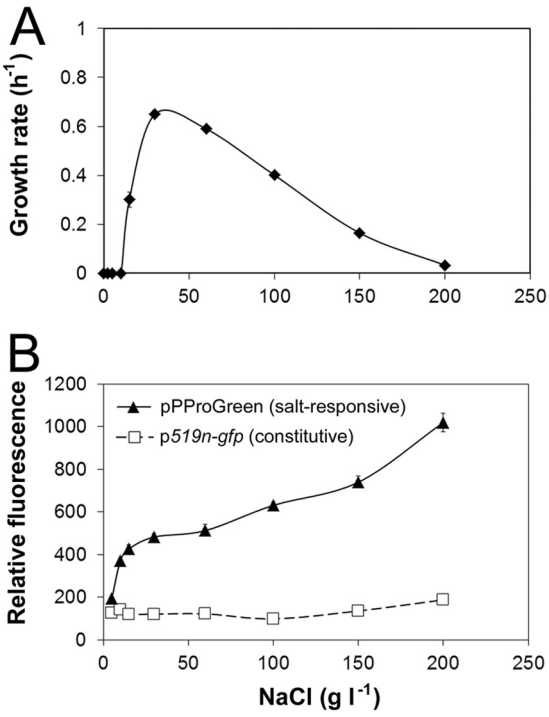

FIG 1 (A and B) Growth rate of *Halomonas variabilis* strain Tx42 (A) and GFP fluorescence of salt-responsive *H. variabilis* (pPProGreen) (triangles) and constitutively fluorescent *H. variabilis* (p519*n-gfp*) (squares) determined by fluorimetry of cells (B) grown in a range of NaCl concentrations *in vitro*. Standard deviations of the mean of three replicate cultures for each point are included but are generally too small to be seen.

abundance on leaves after spray inoculation (such that the leaf surface was lightly moistened but without visible drops), followed by 2 days of high humidity (95 to 100%), and then by 2 days of relatively low ambient humidity (ca. 50%). Because *Tamarix* secretes large quantities of salts and other solutes onto its leaves (4), a hygroscopic environment is created in which the solutes deliquesce into small "dew" droplets when there is sufficient moisture in the air over extended periods of time (Fig. 2A). In contrast, the water droplets quickly evaporate in drier conditions (Fig. 2B) and salt crystals are apparent on the leaf surface (Fig. 2C). *H. variabilis* multiplied over 2,000-fold on *Tamarix* leaves within 48 h of inoculation when the plants were kept at high humidity, reaching an average population size of 1.9×10^7 CFU per gram leaf (CFU g^{-1}) (Table 1), a figure close to that observed in natural *Tamarix* phyllosphere communities (10^6 to 10^7 g^{-1}) (2). However, after plants were transferred from a humid environment to one with low ambient humidity for an additional 48 h, the culturable *H. variabilis* population decreased by >100-fold (to 1.6×10^5 CFU g^{-1}). To estimate their viability, cells recovered from leaves were stained with propidium iodide and examined by fluorescence microscopy; most (~92%) of the GFP-marked cells recovered from humid *Tamarix* leaves had intact membranes, while the majority (~94%) of fluorescent cells associated with *Tamarix* leaves exposed to ambient humidity had permeable membranes.

Halophilic salinity reporter. Both the reduced growth rate of *H. variabilis* at high salinities in culture and its high mortality on dry plants suggested that salinity might affect the distribution of *Halomonas* and other halophiles on *Tamarix* trees. In order to

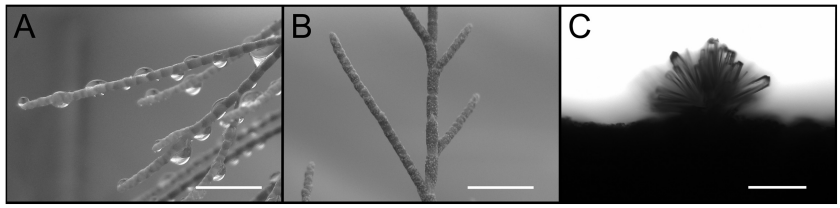

FIG 2 Characteristic environments of the *Tamarix* leaf. (A to C) *Tamarix* leaves in high (100%) humidity with associated water droplets that form in discrete sites on the leaves (A), dry leaves under ambient (50%) humidity (B), and a close-up of the crystallized salt structures that form under ambient humidity (C). Bars, 1 cm (A and B) and 0.1 mm (C).

ascertain the local concentrations of salt that bacteria would experience at any given site on *Tamarix* leaves, we introduced the reporter construct pPProGreen, which contains the salinity-inducible *E. coli proU* promoter region fused to a promoterless *gfp* reporter gene (13), into *H. variabilis* strain Tx42 in order to develop a halophilic salinity bioreporter strain. *H. variabilis* cells carrying pPProGreen were brightly fluorescent in TXM broth media containing 30 g of NaCl liter^{-1}, indicating that the *E. coli proU* promoter was capable of driving *gfp* transcription in this strain. To determine the salt-responsiveness of this bioreporter, the GFP fluorescence of *H. variabilis* (pPProGreen) was measured in cells growing exponentially in modified TXM containing a range of NaCl concentrations. GFP fluorescence exhibited a strong positive linear correlation with salinity, increasing up to 200 g of NaCl liter^{-1} ($R^2 = 0.87$, $P < 0.0001$) (Fig. 1B). Although this salinity-dependent response was similar to that observed previously in *Pantoea* and *Pseudomonas* species (14), it occurred over a much wider range of NaCl concentrations. As a control, the GFP fluorescence of *H. variabilis* harboring p519*n-gfp*, in which *gfp* is constitutively expressed, was also measured in cells grown at different salinities (Fig. 1B). At NaCl concentrations above 100 g liter^{-1}, *H. variabilis* (p519*n-gfp*) also exhibited slightly enhanced GFP fluorescence, likely due to its lower growth rate and thus a slower dilution of GFP. Consequently, although the fluorescence of *H. variabilis* (pPProGreen) is responsive to NaCl concentrations up to 200 g of NaCl liter^{-1}, it should be noted that its relative fluorescence induction, normalized to *H. variabilis* (p519*n-gfp*), is maximized at 100 g liter^{-1} (calculations not shown). This, however, does not detract from its utility to report at high salinities but rather reflects different mechanisms by which it might work; cells harboring pPProGreen could be brightly fluorescent at the highest salinities because of their reduced growth rate instead of a further induction of *proU*.

High salinity has little impact on *H. variabilis* growth in *Tamarix* dew drops. Although cells carrying pPProGreen have been previously utilized to estimate water availability on plant surfaces, fluorescence of the bioreporters was only compared to cultured cells exposed to different salinities and could not be verified by independent measurements *in situ* (13, 21). While our goal was to examine the range of salinities encountered across the entire *Tamarix* leaf surface, the fact that *Tamarix* leaves accumulate semistable deliquesced dew drops of hydrated salts and sugars in high humidity represented a unique opportunity to first test the *in situ* performance of the *Halomonas* bioreporter in these specific leaf niches. We thus inoculated *H. variabilis* (pPProGreen) onto *Tamarix* plants that had been grown both with or without NaCl amendments to the soil and subsequently harvested individual dew droplets 48 h later. Cells within the droplets were subjected to quantitative fluorescence microscopy, and the total dissolved salts in each droplet was measured. In addition, cell counts in each dew droplet were determined by dilution plating. Salinities of individual droplets ranged from 9 to 210 g liter^{-1}, and growth was apparent in all droplets (Fig. 3A) with an average density of ~10^5 CFU μl^{-1} recovered compared to the inoculum density of only 10^3 CFU μl^{-1}. The highest population density was reached in a droplet having only 13 g of dissolved salts liter^{-1}; a salt concentration that would have supported very slow growth, if at all, of *H. variabilis* in shaken broth culture (Fig. 1A). Not surprisingly, much higher dissolved salt concentrations were observed in dew droplets from plants watered periodically with NaCl solutions compared to plants grown without added soil-applied salt (Fig. 3B). This observation supports previous observations that *Tamarix* plants actively transport salts from the roots to leaf surfaces (22). Bacteria grew to high densities in dew drops collected from both salty and nonsalty plants. No significant positive or negative correlations were found between total dissolved salt concentrations and cell counts, either when considering all drops or just those with salinities above or below 30 g liter^{-1} (the concentration at which our *in vitro* experiments suggest we should observe optimal growth). These results suggest that, although salinity might impact the growth rate *in vitro*, under the conditions tested in this experiment, salinity does not appear to be a limiting factor for *H. variabilis* growth in *Tamarix* dew drops.

A positive linear correlation was found between the average fluorescence intensity of individual cells in a dew droplet and the dissolved salt concentration in those drops ($R^2 = 0.43$, $P = 0.008$) (Fig. 3B) when drops from all irrigated plants were considered, indicating that *H. variabilis* (pPProGreen) was an effective salt bioreporter in this highly saline habitat. Culture-grown cells exhibited slightly higher GFP fluorescence than cells on plants that had been exposed to a similar concentration of salts (Fig. 3B). Given that conditions negatively affecting the growth rate of *H. variabilis* may increase the GFP fluorescence of cells and yield overestimates of salinity of a sample, we tested for the magnitude of this effect. We compared the GFP fluorescence of cells from

TABLE 1 Bacterial growth and survival on *Tamarix* plants[a]

Strain	Mean density (log CFU g^{-1}) ± SD		
	Starting density	After 2 days at high humidity	After 2 days at ambient humidity
H. variabilis Tx42	3.8 ± 0.2	7.3 ± 1.0	5.2 ± 0.4
P. alimentarius Tx7	4.1 ± 0.1	6.8 ± 0.3	6.3 ± 0.2

[a] Measurements were taken sequentially, with an initial 2 days of high (95 to 100%) humidity, followed by two additional days at ambient (ca. 50%) humidity. This experiment was repeated at least twice for each of the strains.

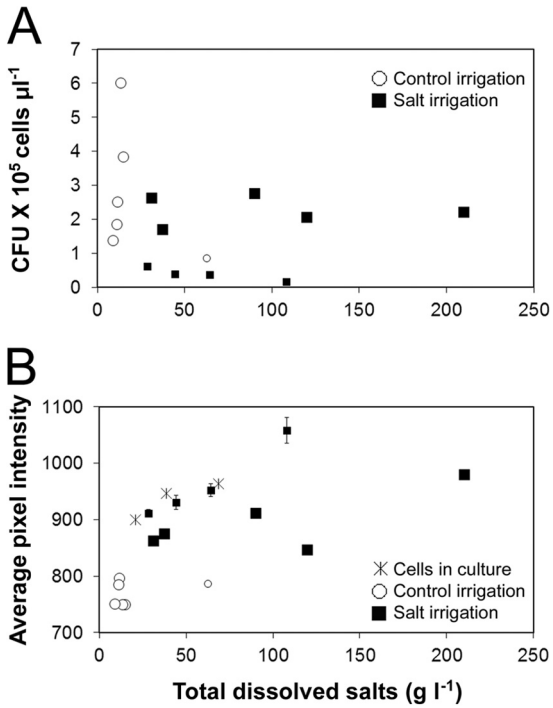

FIG 3 (A and B) Population density (A) and average GFP fluorescence (as determined by quantitative microscopy) of individual cells of *H. variabilis* (pPProGreen) (B) recovered from dew droplets having different salt concentrations from inoculated *Tamarix* plants irrigated with (squares) and without NaCl (circles) as well as from *in vitro* culture (X's, panel B only). Cells were recovered from *Tamarix* leaves 48 h after inoculation with an initial density of 10^3 CFU μl^{-1}. Droplet samples that contained a relatively low population of cells ($<10^5$ CFU μl^{-1}) are represented as smaller symbols. Vertical bars represent the standard error of the mean (B).

droplets that had relatively low cell concentrations ($<10^5$ CFU μl^{-1}) to those with higher concentrations ($>10^5$ CFU μl^{-1}). The majority of droplets that had the lowest concentration of cells also exhibited higher GFP fluorescence than would be expected for the salinity of those drops (small squares, Fig. 3B). Notably, the sample with the lowest cell density (~15,000 CFU μl^{-1}) also had the highest GFP fluorescence (~1,050 average pixel intensity), even though it was not the drop having the highest dissolved salts. When the five droplets with low cell densities were excluded from the analysis, the positive correlation between average GFP fluorescence intensity and salt concentration increased substantially ($R^2 = 0.71$, $P = 0.002$). This supports our hypothesis that factors other than salinity that restrict the growth of *Halomonas* will lead to overestimates of salinity levels.

***Halomonas* oases on *Tamarix* leaves.** Having confirmed that our bioreporter strain reports on a wide range of salinities *in situ*, we next sought to determine the variation and distribution of salinity perceived by *H. variabilis* across *Tamarix* leaves, including microniches found between deliquescent droplets which cannot be tested by independent methods such as conductivity meters. We therefore examined leaf sections by confocal microscopy, viewing cells both from within individual dew droplets as well as those on adjacent regions of the leaf not covered by visible droplets. As we observed before, bacteria were abundant in droplets, and all cells within a given drop had a similar GFP fluorescence (Fig. 4, drops 1 and 2). However, fluorescent bacteria were sparse and of lower average fluorescence (5-fold lower on average, see Fig. 4, insets) in leaf regions that were not immediately covered by dew drops (Fig. 4, leaves 1 and 2a). Many such regions were entirely devoid of fluorescent bacteria (not shown). These results could suggest that either the cells are in regions of such low salts and thus high water availability that their growth is inhibited and *proU* was weakly induced, or, conversely, that they are in desiccated sites and may be dead. Quantification of GFP fluorescence of PI-stained *H. variabilis* (pPProGreen) cells recovered from leaf sections revealed that the presumably dead cells having permeable membranes (ca. 9% of the total cells) formed a distinct population that exhibited on average 25% the level of GFP fluorescence of cells having intact membranes (data not shown). In addition, light dusting of the *Tamarix* leaf surface with powdered fluorescein resulted in dissolution and a resultant fluorescence signature only in punctate regions of the leaf (not shown). Thus, it appears that the regions of the leaf in which deliquescent water drops do not form have very low water availability and do not support the survival of *H. variabilis* to any significant extent. Even under humid

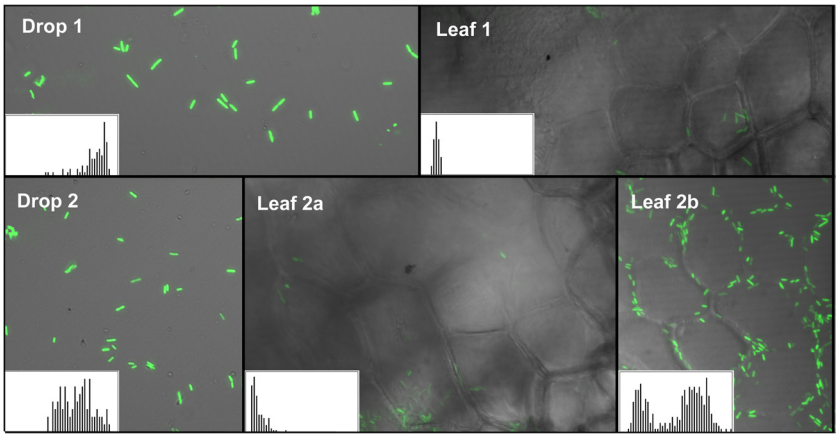

FIG 4 GFP fluorescence exhibited by cells of *H. variabilis* (pPProGreen) recovered from droplets and located in interdrop sites on *Tamarix* leaves. Frequency histograms of GFP fluorescence of individual cells in the image (calculated as average pixel intensity per cell with a range from 0 to 225) are shown in the lower left hand corner of each image.

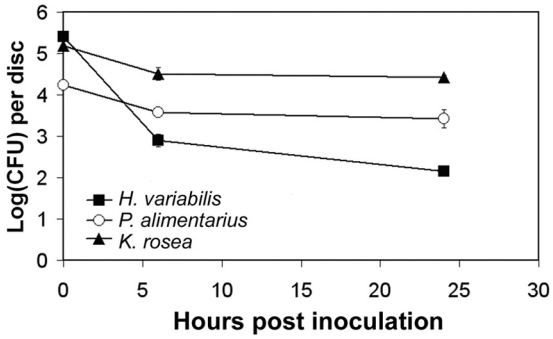

FIG 5 Desiccation tolerance of *Tamarix* bacterial isolates. Strains were inoculated onto paper discs and incubated at 75% humidity. Vertical bars represent the standard deviation of the mean of three replicate cultures for each point. This experiment was repeated twice with similar results.

conditions much of the *Tamarix* leaf surface can be described as a very dry habitat with only punctate wetted oases.

Infrequently, we observed leaf regions not situated directly under dew droplets that hosted relatively large populations of *H. variabilis*; in such instances, the cells were concentrated along plant cell junctions where capillary forces would tend to draw any available moisture (Fig. 4, leaf 2b). Cells in these regions exhibited two distinct fluorescence intensities: some cells were highly fluorescent, while many were dim. The fluorescence intensity of the dim subset of cells was similar to that of the isolated cells on dry leaf surfaces, while the brighter subset of cells in these interdroplet locations were similar to those of cells from within dew droplets. Because the distribution of fluorescence intensities was bimodal even though these populations were physically mixed, it is likely that the dim population represents a subset of cells that have died rather than cells bathed in a lower concentration of solutes.

Comparative life strategies of *Tamarix* colonists. Because *H. variabilis* appears to be constrained by water availability and large regions of the *Tamarix* leaf are apparently inhospitable to its survival even under very humid conditions, we questioned how its osmotic and desiccation stress tolerances might compare with other bacterial strains recovered from *Tamarix* to ascertain whether such taxa may inhabit separate niches on the leaves. Strain Tx7, a fast-growing organism that shares 99% identity with *Psychrobacter alimentarius*, was isolated from *Tamarix* leaves, and Tx52, a slow-growing bright red strain that shares 99% identity with *Kocuria rosea*, was isolated from salt crystals from a *Tamarix* plant. Both strains exhibited growth on TXM plates containing only up to 100 g of NaCl liter^{-1}, substantially less than that tolerated by *H. variabilis*. However, when desiccated on paper filters in 10-μl aliquots of TXM at 75% humidity, the viable population size of *H. variabilis* decreased by >300-fold within 6 h, while both the *Psychrobacter* and the *Kocuria* strains experienced only a 5-fold decrease in culturability in the same time period (Fig. 5). After 18 additional hours of desiccation, the viable population size of *H. variabilis* continued to decrease, whereas no further death of either *P. alimentarius* or *K. rosea* occurred. Despite having a higher salt tolerance, *H. variabilis* was thus much less tolerant to rapid desiccation than these other two species.

The growth and survival of *H. variabilis* and *P. alimentarius* was also compared directly on leaves of *Tamarix* subjected to sequential wet and dry conditions, to test the linkage of desiccation tolerance measured *in vitro* with epiphytic fitness on the salty leaf surfaces. The population size of kanamycin-marked *P. alimentarius* Tx7 increased from an average of 1.3×10^4 CFU g^{-1} to 6.7×10^6 CFU g^{-1} within 2 days on leaves incubated at high humidities (Table 1). Under these same conditions *H. variabilis* achieved a higher population size and its overall level of growth was 5-fold higher, indicating that it is better suited to active growth on humid *Tamarix* plants. However, when exposed to low ambient humidity for two additional days, the culturable population size of *P. alimentarius* decreased only ~3-fold, whereas the population size of *H. variabilis* dropped >100-fold. The higher survival of *P. alimentarius* on dry leaves is consistent with its higher tolerance of desiccation on filters.

DISCUSSION

Previous work revealed that while geographical distance represents a significant barrier to bacterial dispersion to *Tamarix* trees when climatic factors are similar, climatic factors are a major determinant of bacterial community structure on this plant (1, 20). The present study has attempted to determine what environmental factors drive the proliferation of *Halomonas*, a genus exhibiting a strong regional and thus presumably environmental pattern of colonization, on *Tamarix* trees. If the *H. variabilis* strain that we chose as a model *Halomonas* strain is typical of this genus then it appears that it might be considered an opportunistic colonist of *Tamarix*, exploiting leaves under moist conditions independent of salinity levels in the dew drops whose formation is favored by local exudate deposits, but a poor colonist of drier parts of the leaf where its survival is relatively low. Thus, the distribution of *Halomonas* and perhaps other bacteria on *Tamarix* likely is linked to the frequency or duration of the availability of moisture through regular dew-wetting events.

Despite observing a strong influence of salinity on the growth rate of *H. variabilis in vitro*, we did not observe any linkage of salinity to population density in dew drops on leaves. Although we reported that the cell concentration in dew drops was 100-fold higher than the initially applied inoculum, cell growth was certainly more than this amount because it does not account for the increased volume of liquid on leaves as the drop enlarges due to deliquescence; while cells are dividing in drops, they are simultaneously diluted by the absorbance of water from the vapor phase. Since the total bacterial population increased 2,000-fold on the leaf surface over 48 h (Table 1), and the vast majority of the bacteria are present in dew drops, in which their concentration increased an average of 100-fold, then the volume of water that has condensed on the leaf surface would be ~20-fold higher than that initially applied as inoculum. The 2,000-fold growth of *Halomonas* also reflects approximately 11 divisions per inoculated cell, a generation time of about 4 h. This growth rate is much higher than expected from *in vitro* conditions at salinities below 15 or above 100 g of NaCl liter^{-1} (Fig. 1A). It is possible that the high concentrations of dissolved sugars and other substances could enable better growth at both low and high salinities on *Tamarix* leaves than compared with a dilute nutrient medium. Indeed, we observed that even plants not exposed to salty soil, having low salt concentrations in their dew drops, nonetheless harbored dew drops, presumably due to high concentrations of sugars and other hygroscopic substances on these leaves. Alternatively, sampling 48 h after inoculation might have allowed even the slowest growing populations to exhaust the carbon source in a given droplet; sam-

pling at an earlier time point might have revealed a weak correlation between salinity and growth rate.

While this *E. coli*-based *proU* promoter reporter has conferred salt-inducible GFP fluorescence in a number of other bacteria (14), it has not previously been tested in any halophilic organism or at such high salinities. However, we believed this system would function in *Halomonas* since glycine betaine uptake had previously been demonstrated in a *Halomonas* strain (23), and an operon in the sequenced model strain *Halomonas elongata* contains potential homologs to the first two genes in the *E. coli proU* operon. Although the salinity responsiveness of *H. variabilis* (pPProGreen) was clear at low and moderate salinities, at high salinity levels, the increased fluorescence of this bioreporter may be partly due to a slower growth rate (and thus lower GFP dilution) (24). In *H. elongata*, intracellular accumulation of betaine increased with increasing salinity up to 2.5 M (146 g liter^{-1}) NaCl, but decreased slightly at 3 M (175 g liter^{-1}) NaCl (25). While *H. variabilis* (pPProGreen) is, nonetheless, an effective bioreporter up to 200 g of NaCl liter^{-1}, direct assessment of the salt-dependent transcription of its *proU* operon such as with quantitative reverse transcription-PCR might clarify its regulation at elevated salt concentrations.

One of the biggest limitations to whole-cell bioreporters is that their use is constrained to habitats in which the host bacterium can grow and survive (26). Introduction of the *proU*::*gfp* fusion into *H. variabilis* expands the range of salinities that can be examined with such a bioreporter. For measuring salt levels, it is logical to use a strain that can tolerate a wide range of salinities. Unfortunately, dry locations appear detrimental to *H. variabilis*, thus constraining its usefulness to wetted sites, although it is uncertain how useful desiccated cells of any taxon would be as a salinity sensor. Another limitation that is true of most bioreporters is that differences in factors such as carbon sources or the overall metabolic potential of the cells can lead to different reporter gene expression *in situ* than measured with the same stimulus in culture (21, 27, 28). In addition, the concentration of glycine betaine, a compound that is produced by many plants as well as present in rich medium, has been demonstrated to suppress the expression of *proU in vitro* (29). Our observation that bacteria recovered from *Tamarix* leaves exhibited lower fluorescence levels compared to cells grown at similar salinities *in vitro* likely reflects one or more of these factors. For such reasons, it is not appropriate to extrapolate leaf salt concentrations directly from GFP fluorescence measures made in culture.

Despite exhibiting lower salinity tolerance, *Psychrobacter* and *Kocuria* are much more tolerant to desiccation than *H. variabilis*, exemplified both by survival of desiccation on filter papers as well as on plants. Thus, they could be utilizing different life strategies for colonization of saline habitats like *Tamarix* leaves. Some bacterial strains like *H. variabilis* strain Tx42 are capable of extensive growth even in highly saline sites but survive desiccation poorly and thus probably dominate leaves that experience periodic humid conditions, while other bacteria may not grow rapidly on leaves but have superior desiccation tolerance and thus may exhibit long-term persistence on leaves that are often dry. *H. variabilis* might thus be considered to be an r-strategist, while *Psychrobacter* and *Kocuria* have a more k-selected life strategy. Since a given *Tamarix* leaf might be expected to present both wet and dry conditions to bacterial colonists due to the discontinuous nature of water drops that form on its leaves, we speculate that many cells of *Halomonas* may occupy somewhat different sites on a leaf than *Psychrobacter* and *Kocuria* and thus not be direct competitors. Defenses of osmotic and matric stress are often considered to be similar, although a growing body of research has been highlighting the many unique responses bacteria have to these two different stresses (30, 31). For instance, in *E. coli* the compatible solute trehalose, but not glycine betaine, supported desiccation tolerance (32). It should be informative to determine the genes responsible for, and the trade-offs made between, rapid growth, high salt tolerance, and desiccation tolerance in highly saline habitats.

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