Supporting Information

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SI Conclusions

Plant leaves are colonized by an abundance of microorganisms including *Pseudomonas syringae*, which is a model bacterial foliar pathogen and a common resident on plants. *P. syringae* strain B728a is a pathogen of beans and is particularly well adapted for growth both on leaf surfaces and in the apoplast, i.e., the leaf intercellular spaces. This work explores the prediction that leaf-surface, or epiphytic, sites offer a distinct environment for *P. syringae* that differs from apoplastic sites. Bacteria in epiphytic sites likely experience fluctuations in water availability, temperature, and solar UV radiation, as well as frequent nutrient limitation. In contrast, the apoplastic environment may be dominated by plant defense responses that expose bacteria to antimicrobial compounds such as hydrogen peroxide and superoxide. We used global transcriptome profiling of *P. syringae* B728a cells recovered from plants to understand the environmental conditions in epiphytic and apoplastic sites and how the cells respond to these conditions. We compared the transcriptome profiles of cells from plants with those of cells grown in pure cultures and exposed to individual stress conditions predicted to occur in leaf habitats, namely oxidative stress and water, iron, and nitrogen limitation. Our results provide evidence that these two plant habitats offer distinct environments for the bacteria and thus present distinct driving forces for *P. syringae* adaptation.

The transcript profiles of *P. syringae* B728a support a model in which epiphytic sites specifically favor flagellar motility, chemosensing, and chemotaxis (Fig. S8). This model suggests active relocation of bacteria to nutrient-rich sites as well as movement that promotes entry into the apoplast through the stomata. In contrast, the suppression of most chemosensing and chemotaxis genes in the apoplast suggests a relatively uniform nutritional environment in the leaf interior. The transcript profiles support the production of two *P. syringae* surfactants, with synthesis of the surfactant 3-(3-hydroxyalkanoyloxy)alkanoic acid favored on leaf surfaces and likely contributing to swarming motility and with syringafactin synthesis favored in the apoplast. Our results are surprising in their support for an exceptionally high level of phenylalanine catabolism on leaf surfaces. Such catabolism may function to reduce the pool of this critical precursor to phenylpropanoid compounds involved in plant defense, thus suggesting that B728a works to counteract defense responses before its entry into leaves. Also, the transcript profiles suggest that leaf surfaces offer a nutritional environment that promotes active scavenging of foliar phosphate and utilization of plant-derived indole as a source of tryptophan.

Following entry of *P. syringae* B728a into the leaf interior, B728a shifts its expression profile to favor a distinct set of traits. It increases the expression of genes for the degradation of the alternate amino acid GABA. GABA catabolism provides nutritional benefit but also could function in minimizing the repressive effect of GABA on virulence. The expression of biosynthetic genes for secondary metabolites is increased appreciably in the apoplast, thus supporting the known role of the two phytotoxins, syringomycin and syringopeptin, in B728a virulence and suggesting roles for several secondary metabolites. Transcript profiles suggest a major increase in syringolin A synthesis in the apoplast. This metabolite is known to reverse an initial step in plant defense, namely closing the stomata to exclude invading bacteria. High syringolin A gene expression following entry to the leaf interior suggests syringolin A-mediated suppression of additional plant defenses beyond stomatal closure and possibly promotion of stomatal opening to enhance movement back onto the leaf surface after apoplast colonization. Finally, the transcriptome profiles revealed that phage-related genes within the B728a genome are preferentially activated in the apoplast, suggesting opportunities for phage-mediated gene transfer and even pathogen control.

A comparison of the transcriptomes from the *in planta* cells with the transcriptomes of cultured cells exposed to individual stresses provided insights into the environmental stresses that B728a encounters in these leaf habitats. Most notably, cells in both surface and interior sites are clearly limited for water, with the profiles of individual gene transcripts suggesting that cells in epiphytic sites experience greater fluctuations in water availability but less severe shortages of water than cells in the apoplast. This difference was reflected, in part, by the strong expression of genes for compatible solute synthesis, with particularly high expression in the apoplast. In contrast to water, B728a cells on leaves were not limited for iron nor strongly affected by oxidative stress, although they synthesized a set of antioxidant enzymes that were distinct from those made in culture, with particularly high expression in the apoplast. They also were not in a physiological state of starvation as great as that of nitrogen-starved cells in culture, despite the known heterogeneous distribution of nutrients on the leaf surface.

Collectively, these findings provide a rich understanding of the leaf habitats encountered by *P. syringae*, and likely other foliar pathogens, and of the adaptations that *P. syringae* has evolved to exploit these habitats. This knowledge should improve the forecasting of foliar disease based on predicting factors that influence inoculum development and should contribute to strategies for altering pathogen behavior and controlling foliar diseases.

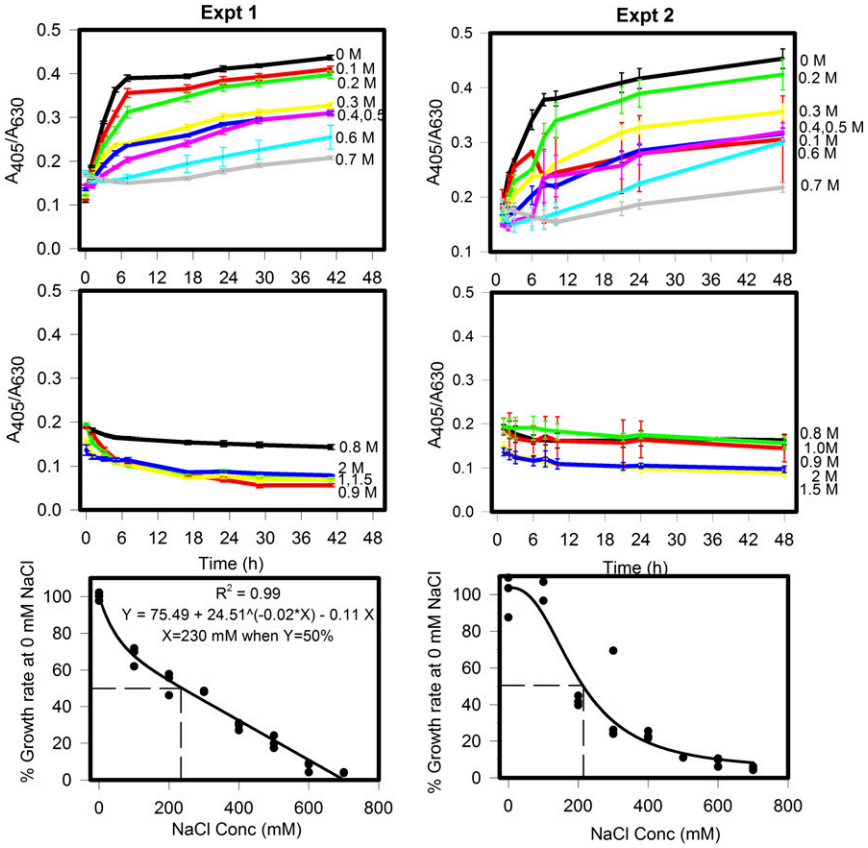

Fig. S1. Evaluation of conditions used to impose osmotic stress on B728a cells. Data for two replicate experiments are shown. Cells were grown in HMM-basal medium, washed twice in HMM-basal medium, transferred to the wells of a microtiter plate, and amended with NaCl to final concentrations ranging from 0.1–2 M. Cell growth was monitored using a microtiter plate reader based on a ratio of the absorbance values at 405 nm (A_{405}) to A_{630}. The growth of cultures amended with 0–0.7 M NaCl (*Top*) or 0.8–2 M NaCl (*Middle*) was plotted. The growth rates during the period of exponential growth were calculated. The percentage growth rate at each NaCl concentration was determined based on the average growth rate of three replicate cultures in the absence of amended NaCl (*Bottom*). In both experiments, a NaCl concentration of ~230 mM resulted in a 50% reduction in growth rate. Values shown in the top and middle plots are means ± SE ($n = 3$). The final protocol for imposing osmotic stress was to amend the cells with NaCl to a final concentration of 230 mM and to incubate them with shaking for 15 min.

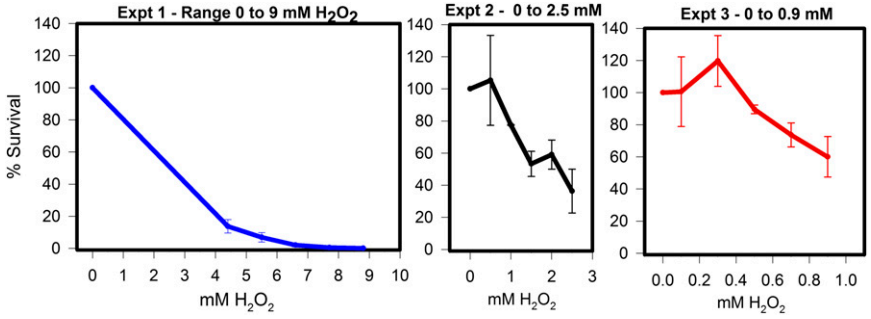

Fig. S2. Evaluation of conditions used to impose oxidative stress on B728a cells. Data are shown for three experiments attempting to identify a sublethal concentration of H_2O_2. Cells were grown in HMM-basal medium, washed twice in HMM-basal medium, transferred to microfuge tubes, and amended with H_2O_2 to final concentrations ranging from 0–9 mM in experiment (Expt) 1 and to increasingly narrower ranges in the subsequent experiments. The cells were incubated for 15 min with shaking; then serial dilutions were plated on King's B agar to enumerate the surviving cells. The percentage of cells surviving the H_2O_2 treatment was determined for each replicate based on the number of cells recovered from the 0-mM H_2O_2 treatment. A previously unopened bottle of 3% H_2O_2 was used for each experiment to minimize H_2O_2 loss from oxidation. Values shown are mean ± SE ($n = 3$). Experiment 3 had our target bacterial density, 5×10^8 cells/mL, whereas experiments 1 and 2 had slightly higher densities. The higher density possibly explains the higher survival rate in the presence of 0.5 mM H_2O_2 in these two experiments, because higher cell densities allow greater cross-protection from secreted catalases. We selected the H_2O_2 concentration of 0.5 mM to use in our studies based on the results in experiment 3. Although this concentration was not technically sublethal (10% of the cells were killed at this level), this concentration represented a good balance between a concentration that was high enough to induce oxidative regulons and one that was low enough to minimize lethality. The final protocol for imposing oxidative stress was to amend the cells with hydrogen peroxide to a final concentration of 0.5 mM and incubate them with shaking for 15 min.

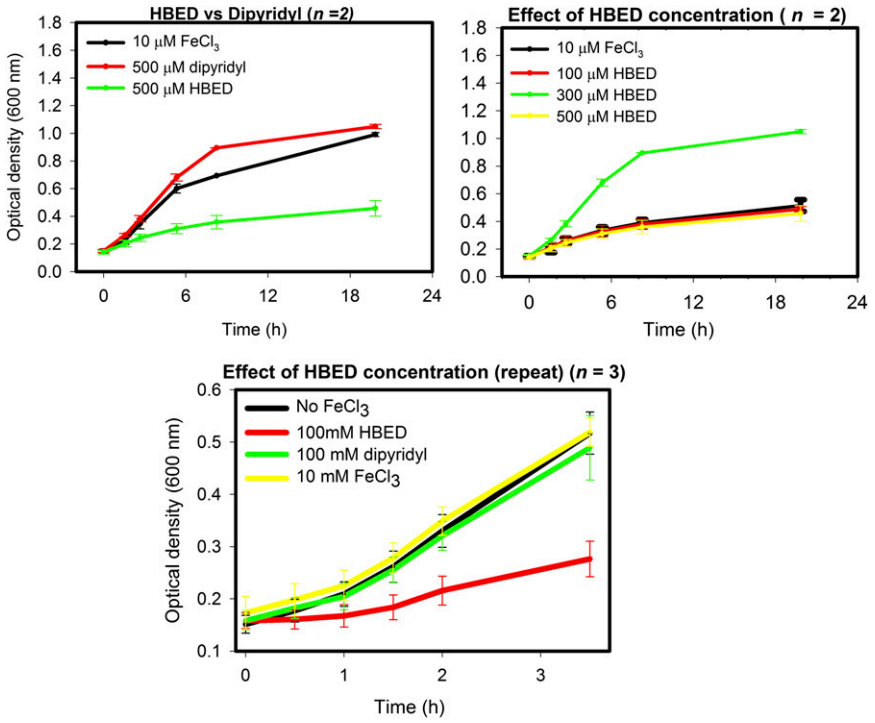

Fig. S3. Evaluation of conditions used to impose iron starvation on B728a cells. Cells were grown in HMM-basal medium, washed twice in HMM-basal medium lacking $FeCl_3$ (HMM Fe), and then transferred into HMM Fe in tubes either lacking iron or containing various concentrations of $FeCl_3$ or the chelators dipyridyl or N,N′-di(2-hydroxybenzyl)ethylenediamin-N,N′-diacetic acid monohydrochloride hydrate (HBED) (Strem Chemicals). Cell growth was monitored based on OD_{600}. The acyl-homoserine lactone was omitted from the HMM and HMM-Fe media here. Dipyridyl was not as effective as HBED at limiting iron. A concentration of 100 μM HBED was sufficient to limit growth. The final protocol for imposing iron limitation was to wash the cells twice in HMM medium lacking an iron source, resuspend the cells in this same medium but containing 100 μM HBED, and incubate them with shaking for 2 h. For 100 mM HBED: 0.0425 g HBED was placed in 1 mL of HMM-basal medium, a partial pellet of KOH was added, and 1 M HCl was added in 10-μL aliquots to adjust the pH to ~8.0 (determined using pH paper). The final concentration was calculated based on the final volume. (Note: HBED does not dissolve in an aqueous solution until it has been alkalinized; then the pH must be readjusted to the desired level.)

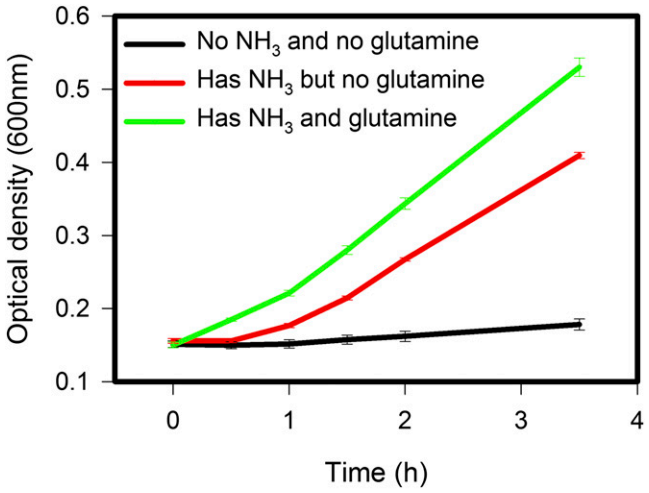

Fig. S4. Evaluation of conditions used to impose nitrogen starvation on B728a cells. Cells were grown in HMM-basal medium, washed twice in HMM-basal medium lacking NH_3 and glutamine, and then transferred into HMM-N in tubes either lacking NH_3 and glutamine or containing NH_3 with or without glutamine. Cell growth was monitored based on OD_{600}. Values shown are the mean ± SE ($n = 3$). Nitrogen limitation was detected within 1 h based on a difference in the growth of cells in the absence and presence of a nitrogen source. The final protocol for imposing nitrogen limitation was to wash the cells twice in HMM medium lacking a nitrogen source, resuspend them in this medium, and incubate them with shaking for 2 h.

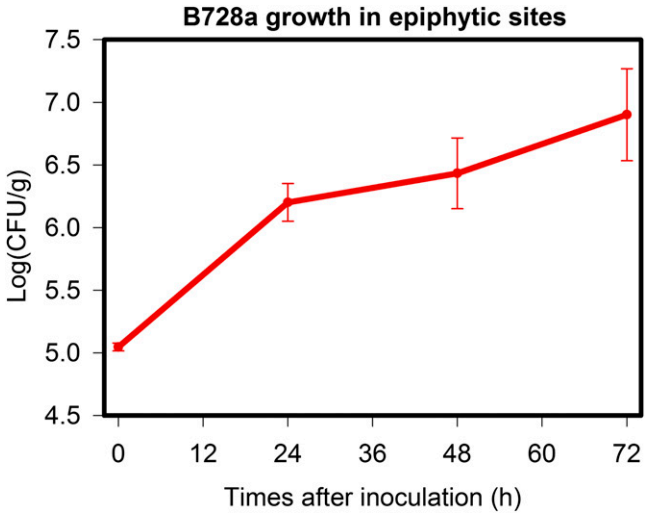

Fig. S5. Evaluation of conditions used to generate epiphytic populations of B728a cells on bean leaves. Cells were grown in HMM-basal medium, washed twice in HMM-basal medium lacking $FeCl_3$, NH_3, glutamine, and *N*-(β-ketocaproyl)-L-homoserine lactone (AHL), and diluted in water containing 0.01% Silwet L-77 to a density of 1×10^6 cells/mL. Plants were grown at a high density (10 plants per 4-in pot) until their primary leaves were fully expanded. They were inoculated with B728a cells by submerging the leaves in 1 L of the inoculum for 20 s, enclosing each pot in a plastic bag to create a tent, and incubating the pots on a laboratory bench at 25 °C for 24 h. The bags were removed, and the plants were incubated at 25 °C for 48 h at the ambient relative humidity. The cells were recovered from four leaves by sonication and were enumerated by plating on King's B agar containing rifampin (50 μg/mL) and cycloheximide (100 μg/mL). The final protocol for collecting epiphytic cells varied from this inoculation scheme as described in *Methods*; most importantly, it was performed at a higher relative humidity to enhance the number of cells recovered from the leaves.

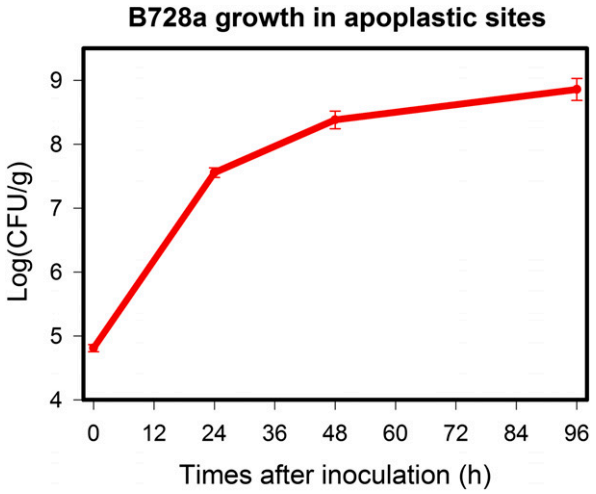

Fig. S6. Evaluation of conditions used to generate apoplastic populations of B728a cells in bean leaves. Plants were grown and inoculum was prepared as described in the legend of Fig. S5. B728a cells were inoculated by submerging the plants in 500 mL of the inoculum, subjecting the submerged plants to a vacuum for ~2 min, and then gently releasing the vacuum and leaving the plants submerged until they were infiltrated with the bacterial suspension. The plants then were removed from the inoculum and allowed to dry on a laboratory bench. The plants were incubated for 48 h under plant growth lights with a 12-h photoperiod. The cells were recovered from eight leaves by homogenization and were enumerated by plating as described in the legend of Fig. S5. The final protocol for collecting apoplastic cells involved this inoculation scheme and sampling at the 48-h time point.

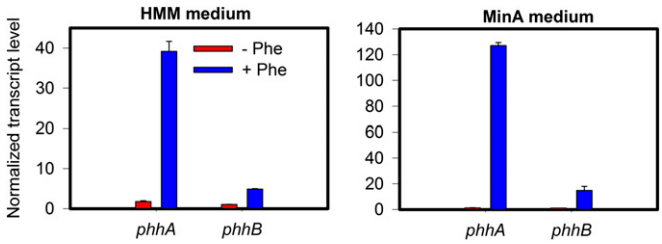

Fig. S7. Effect of phenylalanine on the transcript levels of *phhA* and *phhB*. Cells that were grown to exponential phase in HMM-basal medium lacking AHL and in MinA medium were amended with water (−Phe) or 10 mM phenylalanine (+Phe). Total RNA was extracted after 15 min using the RNeasy mini kit (Qiagen), converted to cDNA using the qScript One-Step SYBR qRT-PCR Kit (Quanta Biosciences), and subjected to quantitative PCR using the primers phhA-F (CGAATTCACCCATACCTACGG), phhA-R (GTAGATTTTCCGCCCTTGTG), phhB-F (AATTGCCGGAACTGCTCA), and phhB-R (CCAGCGCCCATTTGAAATTC). The transcript levels were normalized to the housekeeping gene *hemD* [primers hemD-F (GCACAGCGTTCGATCATTTTC) and hemD-R (TGCTGAACCCACACTGAAC)].

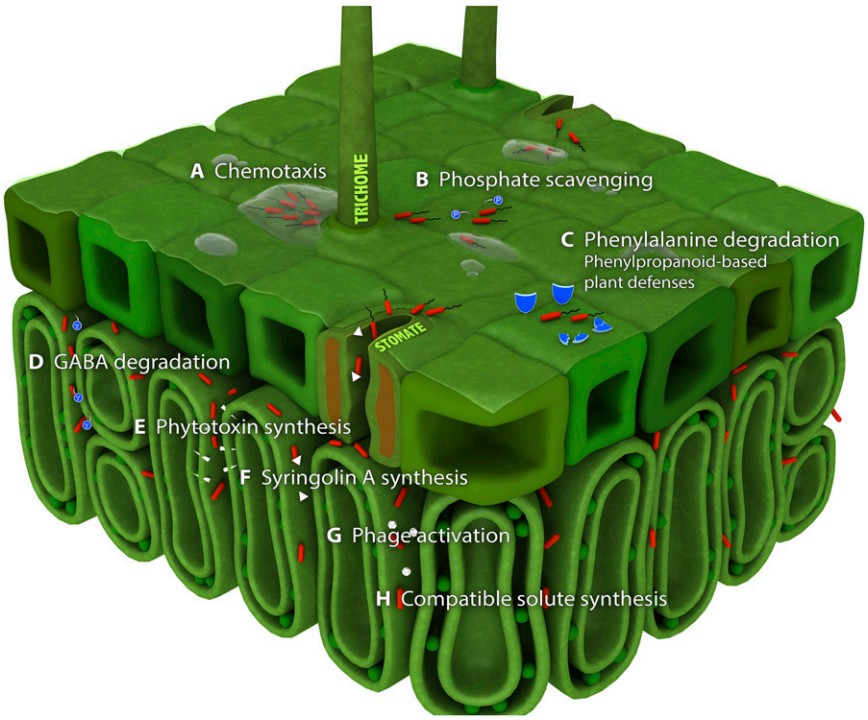

Fig. S8. Leaf cross-section model depicting *Pseudomonas syringae* B728a activities on and within a leaf. Based on transcriptional profiling, B728a cells on leaf surfaces (*A*) are motile and utilize chemosensing and chemotaxis to locate and move toward nutrients and are particularly active in (*B*) scavenging foliar phosphate and (*C*) degrading phenylalanine, which may help counteract phenylpropanoid plant defenses. In contrast, B728a cells within the leaf interior are particularly active at (*D*) degrading γ-amino butyric acid (GABA), (*E*) synthesizing phytotoxins and other secondary metabolites, (*F*) producing syringolin A, a metabolite known thus far only for suppressing host defenses on the leaf surface, and (*G*) activating cryptic phage genes. Lastly, although cells in both locations are water limited, (*H*) cells in the leaf interior expressed genes for compatible solute synthesis at a particularly high level, suggesting greater water limitation in the leaf interior. Blue objects represent plant products; white objects represent substances produced by the bacteria; red rods represent bacteria.

Other Supporting Information Files

Table S1 (DOCX)
Table S2 (DOCX)
Table S3 (DOCX)
Table S4 (DOCX)
Table S5 (DOCX)