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Conditional Survival as a Selection Strategy To Identify Plant-Inducible Genes of *Pseudomonas syringae*

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A novel strategy termed habitat-inducible rescue of survival (HIRS) was developed to identify genes of *Pseudomonas syringae* that are induced during growth on bean leaves. This strategy is based on the complementation of *metXW*, two cotranscribed genes that are necessary for methionine biosynthesis and required for survival of *P. syringae* on bean leaves exposed to conditions of low humidity. We constructed a promoter trap vector, pTrap, containing a promoterless version of the wild-type *P. syringae metXW* genes. Only with an active promoter fused to *metXW* on pTrap did this plasmid restore methionine prototrophy to the *P. syringae metXW* mutant B7MX89 and survival of this strain on bean leaves. To test this method, a partial library of *P. syringae* genomic DNA was constructed in pTrap and a total of 1,400 B7MX89 pTrap clones were subjected to HIRS selection on bean leaves. This resulted in the enrichment of five clones, each with a unique *Rsa*I restriction pattern of their DNA insert. Sequence analysis of these clones revealed those *P. syringae* genes for which putative plant-inducible activity could be assigned. Promoter activity experiments with a *gfp* reporter gene revealed that these plant-inducible gene promoters had very low levels of expression in minimal medium. Based on green fluorescent protein fluorescence levels, it appears that many *P. syringae* genes have relatively low expression levels and that the *metXW* HIRS strategy is a sensitive method to detect weakly expressed *P. syringae* genes that are active on plants. Furthermore, we found that protected sites on the leaf surface provided a higher level of enrichment for *P. syringae* expressing *metXW* than exposed sites. Thus, the *metXW* HIRS strategy should lead to the identification of *P. syringae* genes that are expressed primarily in these areas on the leaf.

While genetic screens have been valuable in the study of habitat-specific microbial processes, no single genetic screen is likely to identify all of the genes expressed in the conditions under study. Primary limitations to these approaches stem from biases inherent to the screening methodology and the properties of the target genes themselves. For example, insertional mutagenesis and its recent derivatives are powerful tools that can identify genes necessary for growth in specific environments (13, 27). However, rather than revealing the full complement of traits, only those genes with large quantitative contributions to whole cell behavior can be identified by these approaches. Genes with incremental or redundant phenotypes are likely to remain cryptic in such mutant screens. These types of genes are important to consider because they often constitute a large portion of the genome (e.g., 90% of known *Escherichia coli* genes are nonessential according to the profiling of the *E. coli* chromosome database [http://www.shigen.nig.ac.jp/ecoli/pec/index.jsp]) and therefore are likely to provide relevant information about the organism's adaptation and growth in the environment under study.

Methodologies that identify genes induced in a habitat- or host-specific manner, irrespective of whether those genes confer an essential phenotype, are complementary to loss-of-function strategies. These screens are based on "trapping" gene promoters in front of a gene conferring an essential and/or easily monitored phenotype. Osbourn et al. were the first to apply this type of positive selection by using chloramphenicol resistance as a selective reporter for *Xanthomonas* genes expressed during invasion of turnip seedlings (30). This approach was later developed into a general screening strategy termed in vivo expression technology (IVET) (25). A variety of related IVET strategies have since been reported, each with different selection criteria. Examples include the habitat- or host-specific complementation of nutritional auxotrophies (25, 31, 39); expression of selectable antibiotic resistance genes (14, 26); irreversible recombination by γδ resolvase in a recombination-based IVET, leading to a genotypic change in cells (8, 9); or differential fluorescence induction (DFI), whereby cells are selected on the basis of enhanced green fluorescent protein (GFP) fluorescence (37, 38). The successes of IVET and related promoter-trapping strategies have recently been discussed in several comprehensive reviews (2, 10, 24, 32). Taken together, these screens have been effective in identifying genes expressed in a host- or habitat-specific manner. Many of these genes were later shown to be not essential and hence would have been missed by mutagenesis approaches. Furthermore, these approaches remain among the most comprehensive for identification of genes upregulated in environments where more recent technologies, such as DNA microarray transcriptional profiling, are presently not practical due to the presence of contaminating organisms and the low number of cells contained in each sample.

Our laboratory has previously applied both negative and positive screens to identify genes expressed by a plant-pathogenic strain of *Pseudomonas syringae* pv. syringae in its pre-

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ferred habitat, the leaf surface or phyllosphere. Random Tn*5* mutants of *P. syringae* with a reduced ability to grow and survive in the phyllosphere, i.e., with a reduced epiphytic fitness, were identified (21, 22). From that study it became apparent that epiphytic fitness is conferred not by a few genes with large individual effects but rather by many genes contributing incrementally to fitness. In a complementary approach to determine how many genes are expressed selectively on leaves and hence might contribute to fitness, a library of *P. syringae* mutants containing random fusions of a promoterless *lux* operon were examined for luciferase activity (11). That study revealed that approximately 2% of *P. syringae* genes are preferentially expressed in the phyllosphere, thus indicating that many traits contributing to epiphytic fitness may not be expressed in culture. These results indicated that many phyllosphere-specific genes might contribute to bacterial adaptations evolved to exploit this habitat. The use of a nonselective reporter such as luciferase, however, proved to be far too laborious a procedure to identify all of the many plant-induced genes in this species.

To efficiently identify *P. syringae* genes that are expressed specifically on leaves and hence have a likely role in epiphytic fitness, we have developed a new variant of the IVET system termed habitat-inducible rescue of survival (HIRS). This selection strategy differs from other IVET approaches in that it is based on the production of an endogenous factor required for complementation of a conditionally lethal phenotype in vivo. In this report, we present a HIRS strategy to enrich for phyllosphere-specific genes of *P. syringae* based on *metXW*, two genes which were previously determined to be cotranscribed from a single, unregulated promoter and which were required for methionine biosynthesis in *P. syringae* (1). Methionine auxotrophs of *P. syringae* have previously been shown to exhibit normal growth on moist plant leaves but were severely reduced in fitness when the plants were subsequently exposed to conditions of low relative humidity (1, 5). We characterize the *metXW* HIRS system, with an emphasis on its strengths and limitations as a genetic screen, and present evidence that this screen is a powerful way to detect weakly expressed genes that are active primarily on leaf surfaces.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Pseudomonas syringae* pv. syringae B728a is a spontaneous rifampin-resistant mutant of a wild-type strain originally collected from bean leaves (23). *P. syringae* B7MX89 is a mutant of B728a containing a Tn3-SPICE insertion in the *metXW* operon (1). The Tn3-SPICE transposon contains a spectinomycin antibiotic resistance marker and is stable in the chromosomes of *P. syringae* strains (20). *P. syringae* cells were routinely cultured at 28°C on King's medium B (KB) (18) or in M9 minimal medium (34) supplemented with 0.4% glucose as a carbon source. Methionine auxotrophs were grown on M9 medium supplemented with 0.3 mM L-methionine (Sigma, St. Louis, Mo.). Plasmids were transferred from *E. coli* DH10B donor cells (Life Technologies) to *P. syringae* by triparental mating with the *E. coli* helper strain HB101(pRK2013) (12). When appropriate, antibiotics were added to the media at the following final concentrations: ampicillin, 150 μg/ml; kanamycin, 50 μg/ml; rifampin, 100 μg/ml; and benomyl, 50 μg/ml.

Recombinant DNA techniques. DNA isolation, PCR, and general recombinant DNA techniques were performed by standard methods (34). Restriction and modifying enzymes were obtained from New England Biolabs (Beverly, Mass.) and Roche Diagnostics Corp. (Indianapolis, Ind.). PCR was performed in a Perkin-Elmer/Cetus (Norwalk, Conn.) DNA thermal cycler. Reaction mixtures of 50 μl contained 50 ng of template DNA, 1 U of *Pfu* polymerase (Promega, Madison, Wis.), and 1 pmol of sequence-specific oligonucleotides (Oligo's Etc., Wilsonville, Oreg.).

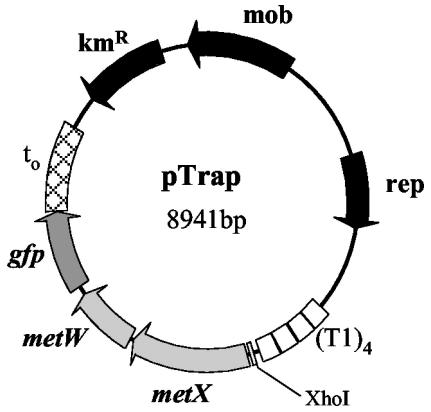

FIG. 1. Schematic diagram of plasmid pTrap, showing the locations of the promoterless *metXW-gfp* transcriptional fusion; the genes required for mobilization (mob), replication (rep), and kanamycin resistance (km^R); and transcription terminators $(T1)_4$ and T_o.

Plant inoculations. Bean plants (*Phaseolus vulgaris* cultivar Bush Blue Lake 274) were grown on greenhouse benches in pots containing three to six seedlings. Once the primary leaves were fully emerged (12 days after planting), the aerial parts of the plants were briefly immersed in 2 liters of 1 mM potassium phosphate buffer (KPB) (pH 7.0) containing suspensions of either a single *P. syringae* strain, pools of *P. syringae* HIRS library clones, or 1:100 mixtures of *P. syringae* B728a and B7MX89. The last mixtures were applied to plants in order to simulate HIRS library pools, whereby it was expected that a maximum of 1 to 2% plant-inducible clones would be present among a predominance of methionine auxotrophs.

After inoculation, the plants were placed in an enclosed chamber maintained at 28°C and kept humid (relative humidity [RH] of near 100%) with water-soaked paper. After 24 h, plants were typically transferred to conditions of low RH (in a growth chamber maintained at 28 to 30°C and 40% RH). To estimate bacterial populations, three to five leaves were collected randomly for each treatment at different times following inoculation. Cells were typically liberated by macerating the leaves individually in 5 ml of KPB with a mortar and pestle. Alternatively, leaves were washed to collect the cells by first placing them into separate tubes containing 20 ml of washing buffer (21), followed by sonication for 7 min in a water bath (Branson Ultrasonics Corp., Danbury, Conn.) and then vortexing for 10 s. For those treatments in which the leaves were both washed and macerated, the leaves were first washed to remove exposed cells and then air dried prior to maceration. Numbers of viable *P. syringae* cells in all leaf samples were determined by plating serial dilutions of the bacterial suspension on the appropriate media by using a spiral diluter-plater (Spiral Systems, Bethesda, Md.).

Population size estimates for bacteria collected from individual leaves were log transformed to achieve normality (16). Mean population sizes among the treatments were compared by using Fisher's unprotected least-significant-difference test with SAS version 6.03 (SAS Institute, Cary, N.C.). This test controls the comparison-wise error rate.

Construction of the promoter-trapping vector pTrap. A 1.8-kb, promoterless version of *metXW* was amplified in a PCR from a cosmid clone containing the entire B728a *metXW* operon (1) by using the primers Mar1 (5′GGATCCTCG AGTAACTAACTAACCTCATTTATGCGAGACAGG3′) and Mar2 (5′GCA GATCTCTGTGGTCCGTCAGCCCG3′). The mar1 primer was designed to include a unique *Xho*I site (CTCGAG) followed by a three-way translational stop sequence (TAACTAACTAA). The PCR product was first cloned into pCR2.1 (TA cloning kit; Invitrogen, Carlsbad, Calif.) for sequence verification and was subsequently removed and ligated into the *Bam*HI site of pPROBE-*gfp*[tagless] (28). Plasmid pPROBE-*gfp*[tagless] contains a promoterless *gfp* gene flanked by T1 terminators and confers resistance to kanamycin. The resulting plasmid was digested with *Sca*I and *Eco*RV and religated to give plasmid pTrap (Fig. 1). This step eliminated the possibility that the *metXW-gfp* cassette could be deleted by recombination between the upstream and downstream copies of the T1 terminators. Plasmid pMTXW is a derivative of pTRAP containing a functional, constitutive *metXW* promoter upstream from the *metXW* locus.

Construction of a partial *P. syringae* genomic DNA library in pTrap. Genomic DNA from B728a was partially digested with *Sau*3AI and size fractionated by agarose gel electrophoresis. DNA fragments 0.8 to 1.0 kb in size were collected by using a Qiaex protocol (Qiagen, Valencia, Calif.) and partially filled in with dGTP and dATP by using the DNA polymerase Klenow (large) fragment (Roche Diagnostics Corp.) (34). The pTrap vector was treated with *Xho*I, and the resulting ends were partially filled in with dCTP and dTTP. This prevents the pTrap vector from self-ligation yet allows the insertion of single fragments with partially filled-in *Sau*3AI ends. The ligated DNA was electroporated into electrocompetent *E. coli* DH10B cells, and transformants were selected on Luria broth containing kanamycin.

Approximately 700 transformants were pooled and used in a triparental mating to transfer plasmids into B7MX89 en masse. For this, exponentially growing cultures of B7MX89, *E. coli* HB101(pRK2013), and DH10B library pools were mixed at a ratio of 4:1:1 and concentrated onto a filter disk which was placed on a Luria agar plate and incubated at 28°C overnight. All cells were then collected from the filter, and appropriate dilutions were plated on KB plates containing kanamycin and rifampin. Transconjugants were replica plated onto M9 medium containing methionine, and at least 3,000 individual recipient colonies were pooled, washed two times in KPB, and prepared for inoculation onto plants.

Restriction digest and sequence analysis of the *P. syringae* library DNA inserts. Changes in the genotypic diversity of the B7MX89(pTRAP) library were monitored by PCR before, during, and after enrichment on bean plants. Library inserts contained in pTrap were amplified with primers Mar3 (5′CAATTGCC AGGAATTGGGGATCGGAAGCTTGC3′) and Mar4 (5′TCGACCCTGTCT CGCATAAATGAGGTTAGTTAGTTAC3′). These primers are complementary to the DNA regions flanking the *Xho*I site. The products were digested with *Rsa*I, an enzyme that recognizes the 4-bp sequence GTAC, and analyzed on a 1% agarose gel containing Tris-acetate-EDTA. Gels were viewed with an Eagle Eye imaging system (Stratagene, La Jolla, Calif.).

The PCR amplification products from those clones that were most highly enriched during HIRS selection on leaves were cloned into the *Sma*I site of pUC18 (34) for sequencing. DNA sequencing was performed at the University of California, Berkeley, Sequencing Facility (mcb.berkeley.edu/barker/dnaseq) with standard M13 forward and reverse primers. Both ends of the cloned PCR products were analyzed, typically allowing us to obtain the entire sequence contained in the chromosomal DNA insert. For gap closure and sequence confirmation, the results from one HIRS library clone were then compared to those from a second clone which exhibited an identical *Rsa*I restriction digest pattern. Basic bioinformatic analysis and annotation were accomplished by using DNAMAN (Lynnon Biosoft, Vaudreuil, Quebec, Canada). Open reading frames (ORFs) were identified by using the ORF finder program (http://www.ncbi.nlm.nih.gov), and BlastX and BlastN functions were used for protein and nucleotide sequence similarity searches. Comparisons to the *P. syringae* pv. syringae B728a genome sequence were made by using the BlastN function at the U.S. Department of Energy Joint Genomes Institute (http://www.jgi.doe.gov).

Determination of GFP fluorescence. Bacterial growth was estimated from the turbidity of cultures at 600 nm with a Perkin-Elmer 3A UV-visible spectrophotometer. GFP fluorescence in the bacterial cultures was determined in a Perkin-Elmer LS50B luminescence spectrometer with an excitation wavelength of 490 nm, an emission wavelength of 510 nm, and a slit width of 8 nm in both cases.

RESULTS

Expression of *metXW* is essential for survival of *P. syringae* on bean leaves exposed to low RH. Methionine auxotrophs of *P. syringae* are severely impaired in their ability to tolerate conditions of low RH on plants (5). This effect was more pronounced when the cells were first allowed to colonize moist plants prior to exposure to low RH (6, 22). To examine this conditionally lethal phenotype in more detail, we compared the epiphytic growth of wild-type *P. syringae* B728a and strain B7MX89, an isogenic *metXW* mutant (Fig. 2). Under conditions of high RH, the epiphytic population sizes of both strains increased at similar rates (g = 3.5 h). When plants were subsequently exposed to low RH, the population size of the strains abruptly declined. While the population size of B7MX89 decreased about 250-fold, only a 50-fold reduction in the numbers of the wild-type strain was observed within 36 h of drying

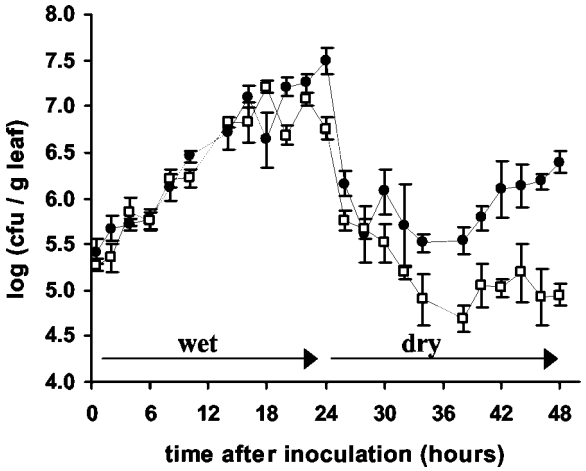

FIG. 2. Population sizes of *P. syringae* strain B728a (●) and B7MX89 (□) on bean leaves exposed to 24 h of high RH followed by 24 h of low RH. The bars represent the standard errors of the means of log-transformed population sizes estimated from washing four leaves at a given sampling time.

of the leaves (Fig. 2). The surviving wild-type *P. syringae* population then increased in size with a generation time similar to that observed when the plants were kept moist. In contrast, the *metXW* mutant was unable to resume growth and, as a result, had a 30-fold lower population size than the parental strain 24 h after exposure to low RH.

Enrichment of Met$^+$ *P. syringae* occurs primarily in protected sites on the leaf. Bacteria liberated by leaf washes (as for Fig. 2) are those that are loosely associated with the leaf or contained in easily reached (exposed) sites, whereas bacteria that are not removed from the leaf by such a procedure and can be released only upon maceration of leaf tissue are considered to reside in protected sites (4). The latter have been proposed to consist of specific microsites on or near the leaf surface and/or within cell aggregates which serve as refuges from exposure to harsh environmental conditions (4). To determine which locations on the leaf provided the strongest enrichment for *P. syringae* with a Met$^+$ phenotype, we compared the population sizes of *P. syringae* B728a and B7MX89 in both areas on leaves during conditions in which the plants were maintained first at high RH for 24 h and subsequently at low RH for another 24 h (hereafter called a wet-dry cycle). Under conditions of high RH, B728a and B7MX89 cells grew primarily within exposed sites on leaf surfaces (Fig. 3). Upon placement of the plants under conditions of low RH, the number of *P. syringae* cells recovered from either site rapidly declined. The *metXW* mutant strain B7MX89 was apparently more sensitive to desiccation stress, however, as its population size decreased 3 times faster in exposed sites and nearly 5.5-fold faster in protected sites than that of the parental strain B728a. Furthermore, there was also no significant increase in the population size of B7MX89 in either protected or exposed sites following imposition of desiccation stress (Fig. 3B). In contrast, the population size of the wild-type strain increased in both locations (Fig. 3A). Protected sites on leaves harbored approximately 10-fold more B728a than exposed sites 24 h after exposure to low RH, suggesting that protected sites were the

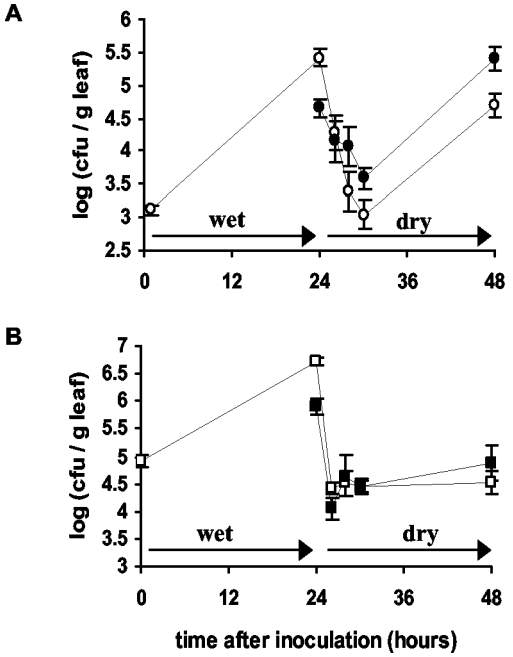

FIG. 3. *P. syringae* B728a (A) and B7MX89 (B) in exposed sites (○, □) and protected sites (●, ■) during a wet-dry cycle. At time zero, cells were found only in exposed sites. Each point represents the mean log-transformed population size ± standard error for five leaf samples.

primary source from which cells with a Met^+ phenotype could be obtained during desiccation stress.

Similarly, cells of B728a preferentially survived desiccation stress in protected sites in leaves even when coinoculated with B7MX89 at a ratio of 1:100 (Fig. 4). This result confirms that even when applied in a mixture containing a great predominance of methionine auxotrophs (a situation similar to what would be expected during the screening of HIRS library pools),

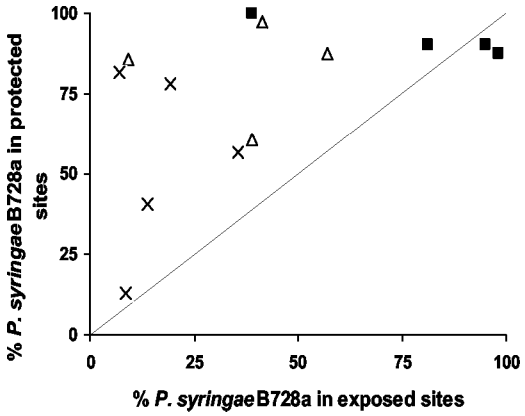

FIG. 4. Relative enrichment of methionine prototrophs from a 1:100 starting mixture of B728a and B7MX89 on bean leaves exposed to different levels of drying stress. After 24 h in high RH, the plants were moved to environments with low (40%) (■), moderate (50 to 60%) (▲), and relatively high (80%) (×) levels of RH for another 24 h. Each point represents a single leaf and the percentage of total B728a cells recovered from the exposed (*x* axis) and protected (*y* axis) sites. The diagonal line represents an equal proportion of B728a cells in both locations on the leaf.

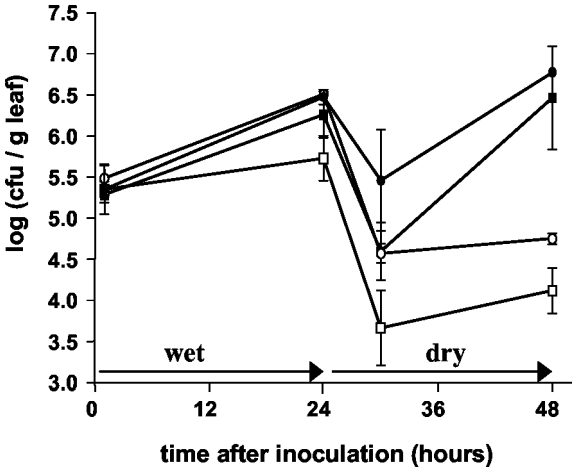

FIG. 5. Population sizes of *P. syringae* strain B728a (●), B7MX89 (○), B7MX89(pTrap) (□), and B7MX89(pMTXW) (■) on bean leaves exposed to a wet-dry cycle. Each point represents the mean log-transformed population size ± standard error for four macerated leaf samples.

Met^+ strains such as B728a are able to survive drying stress and the location of enrichment on the leaf is similar to when the Met^+ strain is applied singly. Furthermore, enrichment in protected sites consistently resulted in at least threefold more wild-type than mutant *P. syringae* cells, independent of whether the plants were exposed to either low, moderate, or relatively high RH following the initial incubation in moist conditions (Fig. 4). In contrast, the total enrichment of wild-type *P. syringae* was directly related to the RH at which the plants were exposed. For example, the *P. syringae* B728a population constituted on average 85% of all *P. syringae* cells recovered from protected and exposed sites on the leaves at the lowest RH (Fig. 4) but constituted only 35% of all *P. syringae* cells recovered from leaves exposed to relatively high RH. These results confirm that a low RH provides the strongest enrichment for *P. syringae* cells expressing *metXW* and that maceration of the leaf tissue enables a higher recovery of these cells from plants than simple leaf washes.

***The metXW* HIRS system enriches for plant-inducible gene promoters.** To determine whether the complementation of methionine auxotrophy by *metXW* was suitable for selection of plant-inducible promoters by a HIRS approach, we constructed the promoter trap plasmid pTrap (Fig. 1). This plasmid contained *metXW* lacking its promoter sequences and thus, when harbored in B7MX89, was unable to restore survival and growth of this strain on plants under conditions of low RH (Fig. 5). In contrast, pMTXW, a variant of pTrap containing the constitutively expressed *P. syringae metXW* promoter, conferred a Met^+ phenotype in culture and restored the ability of B7MX89 to survive on plants (Fig. 5).

We tested the possibility that other *P. syringae* promoters cloned into pTrap could restore methionine prototrophy to B7MX89 and, as a result, permit enrichment for these sequences on plants exposed to a wet-dry cycle. To test this method, we constructed a partial genomic DNA plasmid library by cloning random fragments of *P. syringae* genomic DNA upstream from the *metXW* operon in pTrap. Two sepa-

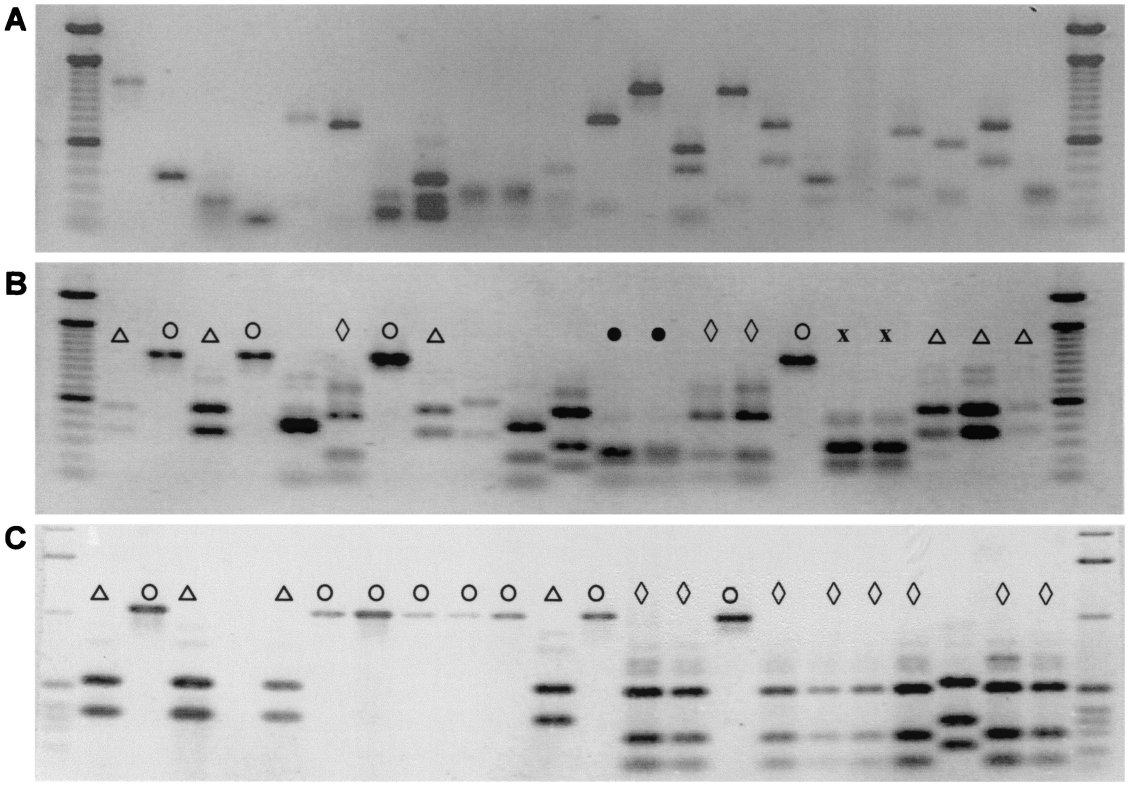

FIG. 6. *Rsa*I restriction digests of PCR products of the genomic DNA library inserts contained in pTrap. Each lane contains the PCR products from a randomly chosen Met$^-$ colony in the B7MX89 pTrap library either prior to selection (A), after a single cycle of selection (B), or after two cycles of selection (C) on leaf surfaces. The symbols (△, ○, ●, X, and ◇) designate a common restriction pattern enriched during the *metXW* HIRS selection.

rate pools of the B7MX89 pTrap library, each containing 700 individual transconjugants, were inoculated onto bean plants and exposed to a wet-dry cycle. *P. syringae* cells were then recovered from leaves by tissue maceration and subsequently examined for the ability to grow in a minimal medium lacking supplemental methionine. Whereas only 20 to 25% of B7MX89(pTrap) cells of the library inoculum exhibited a Met$^+$ phenotype, after a single wet-dry cycle on plants over 60 to 70% of the recovered cells were able to grow in the absence of methionine. The increase in the proportion of methionine prototrophs suggests that there was a strong enrichment on leaves for pTrap clones containing active promoters.

The remaining colonies collected after the wet-dry cycle on leaves had a Met$^-$ phenotype in minimal medium. We can assume that these clones either lacked a promoter or contained promoter sequences conferring only low levels of transcription in culture. If the latter promoters were more active and conferred a Met$^+$ phenotype on plants, they should have been enriched along with the constitutive promoters. To confirm the selection for any such plant-inducible promoters, we monitored the genetic diversity of the DNA inserts contained in the Met$^-$ clones throughout the selection process. Between 20 and 40 colonies randomly collected from each pool before, during, and after two sequential wet-dry cycles on plants were subjected to PCR amplification of their pTrap DNA insert. Restriction analysis of these PCR products revealed that the starting pools contained a high level of diversity among the genomic DNA library inserts (shown for one pool in Fig. 6A). This diversity was reduced to five predominant restriction patterns after a single wet-dry cycle on leaves (Fig. 6B). A second wet-dry cycle in which the inoculum consisted of only those colonies exhibiting a Met$^-$ phenotype in minimal medium resulted in the collection of clones consisting of primarily three restriction patterns (Fig. 6C). In a second library pool that was screened, restriction mapping of the pTrap DNA insert revealed that predominantly two clonal types were enriched (data not shown). These results suggest that there was strong selection for certain clones with a Met$^-$ phenotype in minimal medium; thus, it seems likely that those Met$^-$ clones harbor promoters which are induced during growth on leaves.

Each of the five clones harboring putative plant-inducible promoters (pS16.8, pS16.16, pS17.3, pS17.4, and pS17.15) was inoculated onto bean plants for a wet-dry cycle to compare their population sizes to those of other *P. syringae* strains and library clones. These pS clones grew and survived on plants as well as B7MX89(pMTXW) and five randomly chosen clones with a Met$^+$ phenotype in minimal medium (pProt.4, pProt.5, pProt.13, pProt.14, and pProt.26) (Table 1). In contrast, B7MX89(pTrap) and three clones with a Met$^-$ phenotype chosen randomly from the starting pool (pAux.1, pAux.2, and pAux.3) exhibited much lower epiphytic fitness (Table 1). These results confirmed that the Met$^-$ phenotype conferred by pS clones in culture is complemented in vivo, most logically because of the presence of a plant-inducible promoter se-

TABLE 1. Growth and gene expression characteristics of *metXW* HIRS clones

B7MX89 clone[a]	Population size (log CFU/g of leaf)[b]	Met phenotype in minimal medium	Normalized GFP fluorescence[c]
pTrap	3.8 a	−	16
pMTXW	5.9 efg	+	83
pAux.1	4.3 b	−	NA[d]
pAux.2	4.3 b	−	NA
pAux.3	4.5 b	−	NA
pProt.4	5.5 de	+	46
pProt.5	6.6 h	+	35
pProt.13	5.8 ef	+	157
pProt.14	6.0 fg	+	55
pProt.26	5.3 cd	+	84
pS16.8	5.0 c	−	23
pS16.16	5.7 def	−	27
pS17.3	5.6 def	−	25
pS17.4	5.8 ef	−	28
pS17.15	6.4 gh	−	28

[a] Bean plants were inoculated with B7MX89 containing pTrap, pMTXW, and pTrap library clones unable (pAux) or able (pProt) to complement methionine auxotrophy of B7MX89 in minimal medium. The pS designation indicates a unique clone enriched by the *metXW* HIRS system.

[b] Data are means for three to five leaves exposed to a 48-h wet-dry cycle. Values with the same letter do not differ significantly by the Fisher's unprotected least-significant-difference test.

[c] Average fluorescence value normalized for the cell density (optical density at 600 nm). The coefficient of variation among the GFP fluorescence measurements for each clone was typically 10%.

[d] NA, not available.

quence upstream of the *metXW* locus. In contrast, the low population sizes conferred by pAUX clones and B7MX89(pTrap) itself indicate that their DNA inserts lacked promoter sequences that were active while the cells were on plants.

Sequence analysis of HIRS-selected clones. To facilitate the identification of the plant-inducible promoters among the *metXW* HIRS-selected clones, representatives of each of the five predominant clonal types were selected for sequencing of their pTrap library DNA. Potential ORFs on these DNA sequences were identified, and conceptual translation of these ORFs was used for comparisons to proteins contained in the databases. Sequence analysis of the chromosomal DNA insert contained in clone 16.16 revealed the start of a gene with a predicted product 65% identical to an XerD integrase-recombinase of *P. syringae* pv. maculicola (accession number AAK49554). XerD belongs to the integrase family of site-specific recombinases, which act to resolve dimeric DNA molecules formed during various cell processes, including cell division, plasmid replication, and phage integration (3, 17). Although the precise function of this XerD homologue in *P. syringae* is not known, BlastN searches of the draft *P. syringae* B728a chromosome (http://www.jgi.doe.gov) revealed that this gene is located in an uncharacterized region of the genome containing a collection of putative virulence factors.

The predicted product of a gene initiated in clone 17.15 was most similar to the C4-dicarboxylate transport response regulator DctD (79% identity to *Pseudomonas stutzeri* DctD, accession no. CAC44170). Although *dctD* appears to be constitutively expressed in organisms such as *Rhizobium meliloti* (40), we were unable to find the appropriate conditions in culture medium for expression of the *P. syringae dctD* homologue contained in clone 17.15 (data not shown). Furthermore, the promoter region of the *P. syringae dctD* homologue contained a putative σ^{54} consensus sequence (CTGGGCGG[cgaa]TTG CT), suggesting that this gene is expressed in a manner similar to that of other *P. syringae* σ^{54}-regulated genes, including those involved in virulence (15).

Putative plant-inducible genes were more difficult to identify among the other *metXW* HIRS-selected clones. The *P. syringae* chromosomal DNA sequence enriched in clone 17.4 encoded a putative 146-amino-acid protein that was 28% identical to a hypothetical protein of unknown function in *Burkholderia thailandensis* (accession number AAL47566.1). Clone 16.8 contained a 312-bp ORF for which no significant matches in the DNA and protein databases could be found. Clone 17.1 contained a putative *ppkB* gene encoding a putative protein product that was 64% identical to *Pseudomonas aeruginosa* PpkB (AAD22550). However, this gene was found to be in the opposite orientation with respect to *metXW* in the pTrap plasmid. Although two additional ORFs (450 and 1,008 bp), in the same orientation as *metXW*, were contained in the chromosomal DNA insert of clone 17.1, it is not clear whether these sequences encode expressed transcripts in *P. syringae*.

Relatively low promoter activity is sufficient to confer methionine prototrophy in the HIRS system. The *gfp* reporter gene was included in pTrap as a convenient way to monitor promoter activity from any upstream promoter sequences (Fig. 1). Analysis of the GFP fluorescence of pS clones revealed that the plant-inducible promoters conferred only very low levels of expression in minimal medium, typically only slightly higher than those for B7MX89(pTrap) (16 fluorescence units) (Table 1). In contrast, the pPROT clones, which were Met^+ in culture, exhibited significantly higher levels of GFP fluorescence, ranging from 35 to 157 fluorescence units. However, the lowest GFP fluorescence observed in pPROT strains was only slightly higher than that of the pS clones. This suggests that even very low promoter activity was sufficient to complement methionine auxotrophy in minimal medium.

To determine the threshold level of in vitro complementation of methionine auxotrophy, we examined the GFP fluorescence of 140 library clones. Fluorimeter measurements revealed that GFP fluorescence among the strains varied by over 100-fold (Fig. 7). Those strains with a normalized GFP fluorescence value below about 30 flourescence units were unable to grow in minimal medium lacking methionine. Most of the clones that conferred a Met^+ phenotype exhibited only low to moderate levels of GFP fluorescence (Fig. 7). Often these values were below that conferred by the native *metXW* promoter (83 fluorescence units), which itself is considered to have only a low to moderate level of transcription (1). Apparently, many *P. syringae* promoters confer relatively low levels of gene expression, but this level of activity is adequate to produce sufficient MetXW to complement methionine auxotrophy in the pTrap system. This result implies that the *metXW* HIRS selection strategy is biased toward genes in B728a that have very low expression levels in culture media but which are activated on plants to an extent that permits survival and growth of *P. syringae* during desiccation stress.

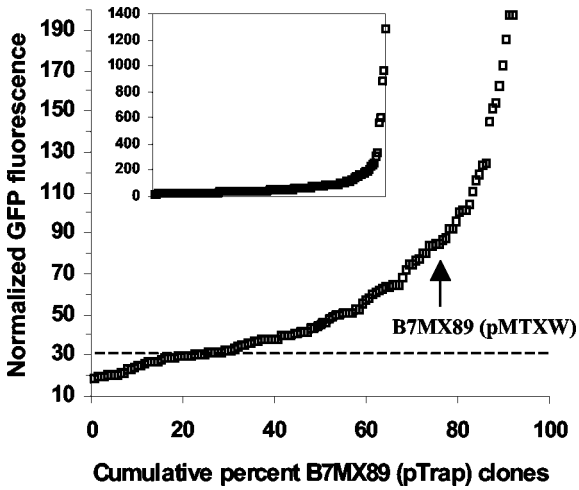

FIG. 7. GFP fluorescence of B7MX89(pTrap) library clones with a Met^+ phenotype in culture medium. A total of 139 individual clones were grown in minimal medium broth containing methionine for 24 h prior to measurement of GFP fluorescence. The average fluorescence value was normalized for the optical density at 600 nm of that clone and plotted according to ascending GFP fluorescence intensity. The internal plot contains the fluorescence values of all clones. The dashed line indicates the lowest value at which methionine prototrophy could be confirmed.

DISCUSSION

The data presented here demonstrate the utility of the *metXW* HIRS strategy to identify genes of *P. syringae* that are induced during colonization of leaf surfaces. The strategy is based on restoration of methionine prototrophy, which, in turn, promotes survival and growth on bean leaves at low RH. Because methionine auxotrophs have a conditionally lethal phenotype on plants, we were able to regulate the timing and extent to which selection pressure was applied. By first exposing the cells to permissive conditions on moist leaves, both Met^+ and Met^- *P. syringae* were able to grow and establish large populations. Upon subsequent exposure to dry, selective conditions, however, only those cells that had accumulated sufficient methionine were likely to be capable of surviving and repopulating the leaf. Although complementation of methionine auxotrophy was specific to this HIRS strategy, the HIRS approach could be easily applied to other systems for which a conditionally lethal phenotype has been identified. As shown in by the *metXW* HIRS example, this approach is advantageous because it is possible to regulate the timing and extent to which selection pressure is applied.

The strength of the *metXW* HIRS strategy is best demonstrated by the decrease in the diversity of pTrap genomic DNA inserts among the *P. syringae* library clones following enrichment (Fig. 6). Following two cycles of enrichment on bean leaves, most colonies with a Met^- phenotype in culture medium represented primarily five different clones, each of which restored wild-type survival and growth to B7MX89 under dry conditions on leaves. We expected approximately 266 clones out of the 1,400 HIRS clones tested in these pools to contain a *P. syringae* promoter oriented properly for transcription of *metXW* (i.e., assuming an average pTrap genomic DNA insert size of 900 bp, random orientation of inserts relative to *metXW*, and an average *P. syringae* gene size of 1 kb). The five unique pS clones with selectable plant-inducible activity therefore constituted 1.8% of those clones that were expected to contain a promoter. This theoretical value is remarkably similar to the frequency of plant-inducible genes which was observed earlier by Cirvilleri and Lindow (11). If this proportion of plant-inducible promoters is an accurate representation of *P. syringae* B728a gene expression patterns, we project that there are at least 120 plant-inducible loci in the 6-Mbp genome of this strain. An efficient selection scheme such as *metXW* HIRS will thus prove to be invaluable in further work to identify the plant-dependent transcriptome of *P. syringae*.

Although the primary aim of this report was to demonstrate the potential of the HIRS approach, we have analyzed the *P. syringae* library DNA sequences that were found to have plant-inducible activity. Sequence analysis of the *metXW* HIRS-selected clones revealed that at least two of the five clones harbored genes in their pTrap DNA inserts for which putative plant-inducible activity could be assigned. These genes, encoding XerD and DctD homologues, are involved in recombination and transcriptional regulation, respectively. Although more thorough analysis of these genes is necessary to determine their function in *P. syringae*, they also highlight regions of the chromosome and transcriptional regulons for which new insights into *P. syringae* adaptations for epiphtyic growth could be found.

The remaining clones contained ORFs for which there are presently no characterized homologues in the databases. This result suggests that bioinformatic analysis alone is not sufficient to identify all plant-inducible genes of *P. syringae* and that any promoter activity initiating from these sequences should also confirmed by independent methods. For example, initial transcriptional activity measurements performed with fusions of the selected pTrap chromosomal DNA inserts to an *inaZ* reporter gene suggest that these sequences confer 5- to 25-fold-higher levels of transcription on plants relative to growth in culture medium (data not shown).

A *gfp* reporter gene was included in pTrap in order to directly assess the relative level of transcriptional activity conferred by *P. syringae* promoters selected by the HIRS procedure. As expected, the plant-inducible promoters selected in this study conferred only very low levels of GFP fluorescence in minimal medium. Alternatively, clones harboring promoters that were active in minimal medium exhibited slight to very high levels of GFP fluorescence (Table 1). All of these clones also had wild-type growth characteristics on plants. Therefore, it is unlikely that high expression levels of GFP are detrimental to *P. syringae* stress tolerance on plants and could interfere with the selection of any highly expressed plant-inducible genes. This result also suggests that complementation of methionine auxotrophy on bean leaves is a qualitative (all-or-nothing) effect. In other words, once *P. syringae* attains a threshold level of methionine biosynthesis (indirectly measured as a GFP fluorescence of over 30 florescence units), the cells are able to adequately respond to desiccation stress and no benefit is gained from a higher threshold level of expression.

The low GFP fluorescence of a collection of pTrap transformants exhibiting a Met^+ phenotype in minimal medium revealed that the overall level of transcriptional activity of most

P. syringae genes is relatively low (Fig. 7). Similarly, it is also likely that only a small fraction of genes of other bacteria are highly expressed in a given environment. For example, Wei et al. found that fewer than 10% of *E. coli* genes represent approximately 50% of all mRNA transcripts when the cells were grown in various media (41). Furthermore, transcripts encoded by at least 20% of predicted and known *E. coli* genes could not be detected reliably by microarray experiments, even when the cells were grown in minimal medium (33, 36, 41). If it is indeed true that the transcriptome of bacteria can be characterized as containing many transcripts with low levels of abundance, then the methionine-based selection strategy will be a powerful way to select for such weakly expressed genes. Because even low levels of *metXW* expression are sufficient to confer a Met^+ phenotype in minimal medium, we should be able to select only those genes that are essentially "off" in culture. In this respect, the high sensitivity of our *metXW*-based HIRS system is probably most similar to that of the recombination-based IVET, which requires only low levels of or transient gene expression to enable selection (9, 19). The types of genes identifiable by these two methods would probably have been overlooked in most other, less sensitive, IVET systems. For example, DFI selection systems are dependent on at least moderate levels of GFP fluorescence to enable selection of induced cells. In our experience, the majority of plant-inducible genes of *P. syringae* found by the HIRS selection did not exhibit sufficient transcriptional activity on plants for the GFP levels to be analyzed by fluorescence microscopy or flow cytometry, yet they could be detected by more a sensitive reporter gene such as *inaZ* (data not shown).

The choice of *metXW* for the HIRS strategy was based upon the previous finding that methionine auxotrophs of *P. syringae* were severely impaired in epiphytic fitness. However, it remains unclear why methionine biosynthesis is critical for epiphytic fitness during the transition to low RH on plants. Methionine can be found on leaf surfaces but only in very small amounts (29, 42). In culture, the growth rate and cell yield of B7MX89 decreased at methionine concentrations of less than 7.5 μg/ml (data not shown). Because B7MX89 grows at similar rates and to similar population sizes as wild-type B728a on leaves at high RH, it appears that at least this amount of methionine is available to cells on leaves. Upon exposure to dry conditions, methionine auxotrophs seem to lack either the metabolic versatility or a specific factor dependent upon methionine biosynthesis to tolerate the transition to dry leaf environments and to resume growth. This defect cannot be complemented on leaves either by cross-feeding with wild-type B728a cells (5, 6) or by topical applications of methionine to the leaves (1, 6). Methionine plays a central role in many biochemical reactions, most prominently in translational initiation and methylation reactions (35). The inability to synthesize methionine could have an impact on one or more of these processes and, consequently, on the expression of traits required for desiccation stress tolerance.

By increasing the level of dryness on leaves, we were able to modulate the enrichment of cells with a Met^+ phenotype. However, irrespective of the stress levels to which plants were exposed, enrichment was consistently greater for cells located in protected sites on the leaf than for cells in more exposed locations (Fig. 4). Calculations of mortality revealed that wild-type *P. syringae* populations in protected sites were apparently less vulnerable to stress than those cells in exposed areas, and the surviving populations were also able to rapidly resume growth. Surprisingly, protected sites did not afford any protection to the *metXW* mutant, and cells quickly succumbed to desiccation in both protected and exposed sites on the leaf (Fig. 3B). One possible explanation for this result is that methionine auxotrophy prevents *P. syringae* from modifying protected sites on the leaf to allow growth during periods of desiccation stress. Nonpathogenic strains of *P. syringae* also fail to tolerate extended periods of desiccation stress on plants (43). These bacteria are apparently unable exploit the interior of the plant for growth as extensively as pathogenic strains and therefore may be more exposed to the more environmentally stressful conditions on the surface of the leaf. As a result of this differential behavior at various leaf sites, the strong enrichment for *P. syringae* with a Met^+ phenotype in mixtures with Met^- *P. syringae* on leaves resulted from both a lack of survival of Met^- cells and the growth of Met^+ cells primarily in protected sites.

Clearly, several factors influence the type and number of genes identified by a promoter trap strategy. The choice of laboratory media and screening approaches set the background limit for those genes considered "off" in vitro. Detection of induction in vivo is dependent on the complementarity of the selectable system to the location, extent, and duration of habitat-inducible gene expression. This effect was illustrated by an IVET strategy recently designed by Boch et al. to isolate virulence genes of *P. syringae* pv. tomato (7). Their approach was based on an in vivo complementation of *hrcC*, a plant-inducible gene required for the type III secretory system. Interestingly, many of the virulence genes identified are in the same regulon as *hrcC*. Genes induced prior to or following the requirement for HrcC may have gone undetected by this scheme. Because pathogenesis occurs only during a discrete phase of the interaction of *P. syringae* with plants, a complementary approach such as *metXW* HIRS should identify an additional set of genes involved in the early steps of colonization and adaptation to the leaf surface. While *metXW* HIRS may not identify all potential plant-inducible traits, its extreme sensitivity, as described by this study, is well suited for identifying those many weakly expressed genes that are overlooked in other IVET procedures. It should be possible to identify all plant-induced genes by coupling the *metXW* HIRS protocol with a complementary approach, such as DFI, which has a lower sensitivity and thus can detect genes with higher basal and induced levels of expression. We are currently performing a thorough application of the *metXW* HIRS system to examine plant-specific gene expression in *P. syringae*. The plant-inducible genes that are identified by *metXW* HIRS are likely to confer incremental contributions toward establishing and maintaining large epiphytic population sizes during the harsh environmental regimens to which plants are typically exposed.

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