The histidine utilization (hut) genes of Pseudomonas fluorescens SBW25 are active on plant surfaces, but are not required for competitive colonization of sugar beet seedlings

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The ability to monitor the spatial and temporal distribution of signals in complex environments is necessary for an understanding of the function of bacteria in the wild. To this end, an existing recombinase-based transcriptional reporter strategy (recombinase-based in vivo expression technology, RIVET) has been extended and applied to the plant-colonizing bacterium Pseudomonas fluorescens SBW25. Central to the project was a rhizosphere-inducible locus, rhi14, which functional analyses show is hutT, a histidine-inducible gene that is required for histidine utilization. A transcriptional fusion between hutT and a promoterless site-specific recombinase (tnpRmut168) results in excision of a chromosomally integrated tetracycline-resistance cassette in a histidine-dependent manner. The dose- and time-responsiveness of the promoterless recombinase to histidine closely mirrored the histidine responsiveness of an identical hutT fusion to promoterless lacZ. To demonstrate the effectiveness of the strategy, the activity of hutT was monitored on sugar beet seedlings. Low levels of transcriptional activity were detected in the phyllosphere, rhizosphere and in plant extract, but not in vermiculite devoid of seedlings. The histidine concentration in the rhizosphere was estimated to be 0.6 μg ml⁻¹. The ecological significance of the hut locus was examined by competing a hutT deletion mutant against the wild-type during colonization of sugar beet seedlings. No impact on competitive fitness was detected, suggesting that the ability to utilize plant-derived histidine is not essential for bacterial colonization.

INTRODUCTION

Bacteria continually alter patterns of gene expression in order to optimize fitness. In complex environments, insight into the moment-by-moment changes in levels of transcription can reveal a great deal about both the genetic and the physiological response of bacteria to their environment and the nature of the environment itself (Merrell & Camilli, 2000; Rainey & Preston, 2000; Lindow & Brandl, 2003). Our long-term goal is to determine the contribution of individual Pseudomonas fluorescens genes to ecological performance (fitness) in the wild (Rainey, 1999; Preston et al., 2001; Gal et al., 2003; Zhang et al., 2004a, b). A first step has been to devise and exploit a genetic strategy that identifies P. fluorescens genes that show elevated levels of transcription in the plant rhizosphere (rhi genes; Rainey, 1999; Gal et al., 2003). These rhi genes are of interest because they are predicted to play a role in the maximization of ecological performance, a prediction recently confirmed for a locus encoding an acetylated cellulose polymer (Gal et al., 2003; Spiers et al., 2003).

Testing this prediction is in principle straightforward. Typically, it involves the generation of defined rhi mutants, which are competed directly with an isogenic wild-type ancestor in the rhizosphere, the ratio of the Malthusian parameters providing a precise measure of the fitness of the mutant relative to the wild-type (Lenski, 1991; Gal et al., 2003). But an important consideration in such analyses is the

Abbreviations: CFC, cetrimide, fucidin and cephalosporin; IVET, in vivo expression technology; Km, kanamycin; Pc, piperillin; RBS, ribosome-binding site; RIVET, recombinase-based IVET; SOE-PCR, splicing by overlapping extension using the polymerase chain reaction; Tc, tetracycline.
heterogeneity of the environment. In a complex environment such as the plant rhizosphere, accurate assessment of the contribution of individual genes to ecological performance requires knowledge of environmental heterogeneity, and especially the spatial and temporal distribution of inducing signals. At present this is not easily achieved. A variety of transcriptional reporters exist, but these tend to yield data that are either too low in resolution (for example, population-level assays based on gene fusions to transcriptional reporters such as 'lacZ' or 'uidA'), or too high in resolution (for example, single-cell analysis of 'gfp expression) to be generally useful. While the analysis of gene expression at the single-cell level is desirable, attempts to determine where and when a particular gene is activated by microscopic analysis of individual cells in a complex 'dirt' environment like the rhizosphere can be 'a needle in a haystack' exercise. We note, however, that bacterial cells to signals in the phyllosphere (Brandl et al., 2001; Leveau & Lindow, 2001). Of use would be strategies to obtain information on specific environmental signals with a resolution between that of population-based assays and that of single-cell reporters such as gfp.

A modification to the in vivo expression technology (IVET) strategy, the recombinase-based IVET (RIVET), provides a possible way forward. RIVET was originally designed to identify infection-induced genes in Vibrio cholerae (Camilli et al., 1994; Camilli & Mekalanos, 1995). It relies on a reporter gene (tnpR) that encodes a site-specific DNA recombinase and an unlinked antibiotic-resistance gene cassette flanked by recombinase recognition sequences (res1). When expressed, the recombinase catalyses the excision of the antibiotic-resistance gene, and the permanent loss of antibiotic resistance acts as a heritable reporter of gene expression. RIVET therefore has the power to detect transiently expressed genes and to monitor gene expression in a small bacterial population. The ability of RIVET to report gene activity in situ was shown by Lee et al. (1999), who used it to study spatial and temporal induction of specific V. cholerae genes in a mouse model. Bacteria were harvested from specific organs at predetermined times and evidence of gene induction was obtained simply from the ratio of tetracycline-sensitive to tetracycline-resistant cells. The resulting plasmid (pAS232) contained a unique HindIII site in the middle of the insert between a truncated ORF of fabG and tRNAAsu (Spies et al., 2002). The res-ltet-res1 cassette (a 3-6 kb Kpn–Pst fragment from pGH436) was cloned into pAS232, which was digested with HindIII and blunted-end filled with the Klenow fragment of DNA polymerase. The whole insert was then PCR-amplified using the general M13 forward and reverse primers containing SpeI restriction sites. To generate the final delivery plasmid pIVET-Tc4, the PCR products were cloned onto the unique SpeI site of pIVETP1, a derivative of the IVET vector pIVETP (Rainey, 1999) with the Tc-resistance gene removed. Plasmid pIVET-Tc4 was mobilized into P. fluorescens SBW25 by conjugation with the help of pRK2013 (Tra+). Integration by single homologous recombination was selected on M9 plates supplemented with CFC, Tc and X-Gal (40 μg ml−1). Allelic-exchange mutants were selected as previously described (Gal et al., 2003) by growing the purified transconjugant for two successive 24 h periods in LB broth without antibiotic selection. The mutant SBW25Tc (SBW25::res1-tet-res1) was confirmed by the loss of the plasmid-encoded Pc resistance.

Transcriptional hut RIVET fusion plasmids were generated from pIVET5, which contained the wild-type tnpR reporter, and its two derivatives with a modified tnpR ribosome-binding site (RBS) (tnpRmut168, tnpRmut133) (Lee et al., 1999). The plasmid vectors were firstly modified by replacing the lacZ gene with a Km-resistance cassette, which was later used as a selective marker for plasmid conjugation. The Km+ cassette was obtained from pUC4K (accession no. X06404) by PCR amplification using primers 4KF and 4KR (Table 1). A KpnI restriction site was incorporated into the PCR primers to facilitate cloning into pIVET5 and its two derivatives, to generate p674Km, p675Km and p676Km. The 2.47 kb hut fragment was then cloned into the SpeI site of these three plasmids to generate the final constructs p674Km-hutT, p675Km-hutT and p676Km-hutT, which have the hutT gene fused to tnpRmut168 and tnpRmut133.

METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Escherichia coli strains were grown on Luria–Bertani (LB) medium (Sambrook et al., 1989) at 37 °C. P. fluorescens SBW25 and its derivatives were grown at 28 °C in LB or minimal medium M9 (Sambrook et al., 1989). Where appropriate, antibiotics were added to the following concentrations (μg ml−1): streptomycin (Sm), 100; tetracycline (Tc), 10; pipercillin (Pc), 150; kanamycin (Km), 50. M9 plates containing half-strength CFC (cetrimide, fucidin and cephalosporin) supplement from Oxoid were used to select for P. fluorescens recovered from the phytosphere of sugar beet.

DNA manipulations. Plasmid DNA was extracted using the Qiagen miniprep extraction kit. DNA restriction and modification enzymes and T4 DNA ligase (New England Biolabs) were used according to the manufacturer’s protocol. DNA fragments from agarose gels were extracted and purified using the Qiagen QIAquick Gel Extraction kit. All oligonucleotide primers (Table 1) were obtained from MWG Biotech. PCR reactions using Taq DNA polymerase (Qiagen) were performed according to the manufacturer’s protocol with an annealing temperature of 58 °C. DNA was sequenced using the BigDye Terminator Sequencing kit (Applied Biosystems) on an Automated DNA Sequencer, model 310 (Perkin Elmer).

Plasmid and strain construction. To integrate the res1-tet-res1 cassette into the SBW25 genome, a previously described two-step allelic exchange method was used (Gal et al., 2003). The delivery plasmid pIVET-Tc4 was constructed by placing the res1-tet-res1 cassette in the middle of a 2.5 kb Kpn–PstI fragment located upstream of the well-characterized wss operon (Spies et al., 2002). The KpnI–PstI fragment had been previously cloned into pBluescript (SK+). The resulting plasmid (pAS232) contained a unique HindIII site in the middle of the insert between a truncated ORF of fabG and tRNAAsu (Spies et al., 2002). The res1-tet-res1 cassette (a 3-6 kb Kpn–Pst fragment from pGH436) was cloned into pAS232, which was digested with HindIII and blunt-end filled with the Klenow fragment of DNA polymerase. The whole insert was then PCR-amplified using the general M13 forward and reverse primers containing SpeI restriction sites. To generate the final delivery plasmid pIVET-Tc4, the PCR products were cloned onto the unique SpeI site of pIVETP1, a derivative of the IVET vector pIVETP (Rainey, 1999) with the Tc-resistance gene removed. Plasmid pIVET-Tc4 was mobilized into P. fluorescens SBW25 by conjugation with the help of pRK2013 (Tra+). Integration by single homologous recombination was selected on M9 plates supplemented with CFC, Tc and X-Gal (40 μg ml−1). Allelic-exchange mutants were selected as previously described (Gal et al., 2003) by growing the purified transconjugant for two successive 24 h periods in LB broth without antibiotic selection. The mutant SBW25Tc (SBW25::res1-tet-res1) was confirmed by the loss of the plasmid-encoded Pc resistance.

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Table 1. Bacterial strains, plasmids and oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Strain, plasmid or primer</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><strong>P. fluorescens</strong></td>
<td></td>
<td></td>
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<tr>
<td>SB25</td>
<td>Wild-type strain isolated from phyllosphere of sugar beet</td>
<td>Rainey &amp; Bailey (1996)</td>
</tr>
<tr>
<td>SB25Tc</td>
<td>SB25::res1-tet-res1</td>
<td>This work</td>
</tr>
<tr>
<td>pI122</td>
<td>IVET fusion strain for pIVETP::hutT</td>
<td>Rainey (1999)</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
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</tr>
<tr>
<td>DH5zypir</td>
<td>supE44 ΔlacU169 hsdR17, recA1 endA1 gyrA96 thi-1 relA1, jpir</td>
<td>Hanahan (1983)</td>
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<tr>
<td><strong>Plasmid</strong></td>
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<tr>
<td>pRK2013</td>
<td>Helper plasmid, Tra+, Km'</td>
<td>Ditta et al. (1980)</td>
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<tr>
<td>pUIC3</td>
<td>Universal IVET vector with 'lacZ, Mob+', Tc'</td>
<td>Rainey (1999)</td>
</tr>
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<td>pIVETP1</td>
<td>Derivative of pIVETP without the Tc' gene</td>
<td>This work</td>
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<td>pUIC3 containing hutT deletion fragment, Tc'</td>
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<td>pAS232</td>
<td>pBluescript containing 2-5 kb Kan-Ps1 fragment from SB25</td>
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<td>pIVET-Tc4</td>
<td>pIVETP1 carrying the res1-tet-res1 cassette in the middle of 2-5 kb fragment from pAS232</td>
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<tr>
<td>p674Km</td>
<td>Derivative of pIVET5, lacZ was replaced with Km' gene</td>
<td>This work</td>
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<td>p675Km</td>
<td>Derivative of pIVET5 with tnpRmut168, lacZ was replaced with Km' gene</td>
<td>This work</td>
</tr>
<tr>
<td>p676Km</td>
<td>Derivative of pIVET5 with tnpRmut135, lacZ was replaced with Km' gene</td>
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<td>p674Km-hutT</td>
<td>p674Km carrying 2-47 kb hut fragment, hutT::tnpRmut168 fusion</td>
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<td>p675Km-hutT</td>
<td>p675Km carrying 2-47 kb hut fragment, hutT::tnpRmut135 fusion</td>
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<td>4K</td>
<td>5′-GGGTAGTACCCCTTTGCCAGTCCGACGTTGT-3′</td>
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<td>PhutT-2R</td>
<td>5′-GGGATGTCAAGCAGCGCGCGGAGTGTG-3′</td>
<td>This work</td>
</tr>
</tbody>
</table>

*Restriction sites incorporated into the primers are underlined. Random complementary sequences designed for the SOE-PCR are shown in lower-case type.

respectively. They were transferred into P. fluorescens SB25Tc by conjugation with the help of pRK2013. Transconjugants were selected on M9 plates supplemented with Km, Tc and half-strength CFC.

Deletion of the hutT was achieved via splicing by overlapping extension using the polymerase chain reaction (SOE-PCR; Horton et al., 1989) in conjunction with a two-step allelic exchange strategy, as previously described (Gal et al., 2003). Briefly, DNA fragments (~900 bp) flanking both sides of the hutT were amplified from SB25 cells by using the two primer pairs PhutT-1F/PhutT-1R and PhutT-2F/PhutT-2R (Table 1). The two DNA fragments were then ligated together by a third PCR reaction using primers PhutT-1F and PhutT-2R. This was possible because of the complementary sequences incorporated into primers PhutT-1R and PhutT-2F (Table 1). The resulting 1-8 kb fragment was first cloned into pCR8 (Invitrogen) and the nucleotide sequence obtained. The fragment was then cloned into the integration vector pUIC3 at the unique BglII restriction site to generate pUIC3AhutT. To construct the hutT deletion mutant of SB25, plasmid pUIC3AhutT was mobilized into P. fluorescens SB25 by conjugation with the help of pRK2013. Integration into the chromosome by a single homogeneous recombination event was selected for on LB agar containing nitrofurantoin (100 μg ml⁻¹), Tc and X-Gal. To select for allelic exchange mutants, single blue-coloured transconjugants were grown for two successive 24 h periods in LB broth without antibiotic selection. The bacterial cells were plated on LB agar containing X-Gal. White Tc' colonies were checked for loss of hutT by PCR amplification using primers PhutT-1F and PhutT-2R.

**Enzyme assays.** In experiments that identified histidine as the inducer, β-galactosidase activities were measured by the standard Miller assay (Miller, 1972) and values were expressed as Miller units. Other β-galactosidase activities were assayed using a more sensitive method that uses 4-methylumbelliferone-β-D-galactoside (4MUG) as the enzymatic substrate. The product (7-hydroxy-4-methylcoumarin,
4MU) was detected in a Hoefer DNA Quant 200 fluorometer (Pharmacia Biotech) following the manufacturer’s instructions. The reaction was monitored at 460 nm with an excitation wavelength of 365 nm. The enzyme activity was expressed as μM 4MU min⁻¹ cell⁻¹.

For in vitro growth assays of the TnpR activities, the hutT::tnpR fusions were first cultivated in LB broth containing Tc (10 μg ml⁻¹). The overnight culture was washed once with sterile distilled water to remove the antibiotic and then 10 μl (~10⁶ cells) was inoculated into 10 ml M9 broth with and without inducer. The level of resolution was measured by plating onto M9 plus Km plates for single colonies. Tc² strains were identified by replica-plating the colonies onto M9 plates containing 10 μg Tc ml⁻¹. Approximately 300 colonies were counted for each treatment.

Plant experiments. Coated seeds of sugar beet (Beta vulgaris var. Amethyst) were germinated and cultivated as previously described (Rainey, 1999) in 5 ml scintillation vials using non-sterile vermiculite as a growth substrate. For experiments testing expression of RIVET fusions, the inoculants were prepared by growing hutT::tnpR fusion strains in M9 broth with Tc, and the overnight cultures were washed in sterile distilled water. About 10⁵ bacterial cells were inoculated onto each seed. Bacteria from the shoot and rhizosphere (roots with attached vermiculite) were recovered on plates of M9 supplemented with Km and half-strength CFC. The proportion of Tc² c.f.u. was measured subsequently by replica-plating the colonies onto M9 plates supplemented with Tc.

Plant extracts used in this study were prepared from the shoot of a one-month-old pot-grown sugar beet. Twenty grams were cut and put into a 50 ml plastic tube containing glass beads and 10 ml distilled water. After vortex-mixing at the highest speed for 10 min, the supernatant was filter-sterilized. The extract was used at one-tenth strength in minimal M9 medium.

The plant competitive colonization assay was carried out with 10 independent cultures. To ensure that strains in experiments were physiologically equal, both the mutant (SBW25ΔhutT) and the wild-type competitor (SBW25-lacZ) were inoculated directly, employing bacteria that had been stored at −80°C, grown in LB broth, and then subcultured once in M9 broth. To initiate the competition, mutant and wild-type from overnight M9 cultures were mixed equally and then washed once in sterile water. The bacterial suspensions were used to inoculate sugar beet seeds as previously described (Rainey, 1999), and initial frequencies of competing strains were determined by dilution plating onto LB plus X-Gal plates. Two weeks after sowing, plants were harvested, and frequencies of the mutant and the wild-type competitor were counted on M9 plates supplemented with half-strength CFC and X-Gal. The initial and final frequencies were then used to calculate the selection rate constant (SRC), which expresses the amount of change in competitive performance relative to the wild-type (Lenski, 1991). In order to factor out any effect of the lacZ marker gene on competitive performance, competition experiments were performed between SBW25-lacZ and the ancestral SBW25 as a control.

**RESULTS**

**rhi14 is histidine inducible and required for histidine utilization**

In order to tailor the RIVET strategy for *P. fluorescens* it was necessary to identify a *P. fluorescens* locus that was plant inducible, ideally in response to a stimulus that could be applied in the laboratory. A search for likely candidates among previously isolated hut fusions revealed rhi14 (in fusion strain PI122), which shows significant similarity to both the histidine transport gene (hutT) of *Pseudomonas putida* and the proline transport gene (proY) of enteric bacteria (Rainey, 1999).

To test whether rhi14 is amino acid responsive, the fusion strain PI122 was grown overnight in minimal medium (M9) in the presence of 10 μg Casamino acids ml⁻¹, and β-galactosidase activity determined using the chromosome-integrated promoterless lacZ reporter carried in the IVET vector (Rainey, 1999). rhi14 showed an approximately 2.5-fold induction compared to the same strain grown in M9 without Casamino acids (data not shown), prompting a more precise analysis of inducing conditions. Fig. 1 shows the response of rhi14 to five biochemically diverse amino acids (histidine, proline, arginine, aspartate and tryptophan). Only histidine was able to activate transcription of promoterless lacZ in the PI122 fusion strain. Further analysis showed that induction by histidine was both dose and time dependent (data not shown).

With the genome sequence of *P. fluorescens* SBW25 now available, the genomic context of rhi14 was ascertained (http://www.sanger.ac.uk/Projects/P_fluorescens). As shown in Fig. 2, rhi14 is hutT, one of 12 genes that are organized in the same orientation and encode enzymes with predicted roles in histidine utilization (hut). IVET fusion strain PI122 contains a transcriptional fusion between hutT and the promoterless ‘panB·lacZ’ genes of pIVETP (Rainey, 1999). The fusion junction is at the 11th nucleotide from the ATG start of hutT. In silico analysis of the hut genes shows that the histidine-responsive promoter is located in front of hutU: expression of the hut genes in *Pseudomonas* is known to be controlled by the HutC repressor (Hu et al., 1989). Details of the locus are shown in Fig. 2.

To obtain experimental evidence for a functional role of hutT in histidine utilization, an in-frame deletion mutant of...
hutT was generated by allelic exchange. If the function of this gene is histidine utilization, then a deletion mutant should not be able to grow on histidine as a sole carbon and nitrogen source. As expected, SBW25ΔhutT grew normally on minimal M9 medium with glucose and ammonia and on minimal M9 with proline as a sole carbon and nitrogen source, but was incapable of growth when histidine was the sole carbon and nitrogen source (the wild-type grew normally on minimal M9 medium with histidine as a sole carbon and nitrogen source). A full description of the regulation, function and organization of this operon in SBW25 will be published elsewhere.

Application of RIVET to *P. fluorescens* SBW25

The RIVET strategy comprises two components: (1) a suicide integration vector pIVET5 (Camilli & Mekalanos, 1995) containing a promoterless version of *tnpR*, which encodes the site-specific recombinase, resolvase, from Tn5; (2) a *res1-tet-res1* cassette, which is the substrate for the recombinase. When *tnpR* is expressed in a strain containing the artificial resolvase substrate cassette, excision (resolution) of the Tc gene occurs, resulting in Tc² daughter cells.

Development of the RIVET strategy for *P. fluorescens* SBW25 began with modification of pIVET5: promoterless lacZY was replaced with a Km-resistance cassette to provide a selectable marker for conjugation. Next, the *res1-tet-res1* cassette was integrated into the *P. fluorescens* SBW25 genome by double homologous recombination (see Methods). The 2.47 kb *hut* gene fragment from IVET fusion PI122 was then introduced into the Km-resistance derivative of pIVET5 immediately upstream of the promoterless recombinase. Finally, the completed *hut::tnpR* fusion plasmid was introduced into *P. fluorescens* SBW25::*res1-tet-res1* by conjugation in which integration by single homologous recombination was selected by screening for Km resistance.

To check whether the recombinase was functional in *P. fluorescens* and capable of resolving the Tc² marker in the *res-tet-res1* cassette, the *hut–tnpR* fusion strain was grown overnight in minimal M9 medium in the presence and absence of 100 μg histidine ml⁻¹. The following morning the cultures were diluted and the cells plated on minimal M9 medium. Approximately 300 colonies from each treatment were replica-plated onto minimal M9 medium containing Tc to determine the frequency of Tc² colonies. The majority of colonies (>90%) from populations grown in the presence of the histidine inducer were Tc². However, a similar proportion of cells grown in the absence of the inducer were also Tc². This result showed that while the recombinase was active in *P. fluorescens*, it was too sensitive to be of general use.

Previous work (Lee et al., 1999) has shown that activity of the recombinase needs to be ‘tuned’ to the strength of the promoter under study; tuning being achieved by modification of the RBS of the resolvase. Given that resolution of the Tc cassette occurred in more than 90% of the cells carrying the *hut–tnpR* fusion, even in the absence of histidine, a less efficient RBS was required. We therefore took advantage of two derivatives of pIVET5 that contain modifications of the RBS of the *tnpR* gene: *tnpRmut168* and *tnpRmut135*. In *V. cholerae*, both *tnpRmut168* and *tnpRmut135* cause a reduction in the translational efficiency of *tnpR* (Lee et al., 1999). Into both *tnpRmut168* and *tnpRmut135* we cloned the 2.47 kb *hut* fragment upstream of the modified *tnpR* and then introduced each into *P. fluorescens* by conjugation and homologous recombination. Modified strains were grown overnight in the presence or absence of 100 μg histidine ml⁻¹ and the ratio of Tc² to Tc was determined by replica-plating. In the presence of histidine, ~45% of cells carrying the *hutT::tnpRmut168* fusion were Tc², whereas all colonies carrying the *hutT::tnpRmut135* fusion retained their Tc² phenotype. No Tc² cells were detected in the absence of histidine.

The utility of *hutT::tnpRmut168* to report histidine availability was more rigorously examined by determining its responsiveness to histidine concentrations spanning four orders of magnitude. In parallel, we examined the histidine responsiveness of an isogenic strain carrying a *hutT::lacZ* fusion. The correspondence between the responses of the two reporters (promoterless *tnpR* and *lacZ*) to increasing doses of histidine was highly comparable (Fig. 3). A similar experiment examining the response of the two reporter strains to 500 μg histidine ml⁻¹ over an 8 h period also yielded highly similar time–response curves (Fig. 4). These data demonstrate the utility of the RIVET strategy in *P.
**fluorescens.** They also show that the level of resolution is dependent on the strength of the inducing signal.

**hutT activity in the plant environment**

With the **hutT::tnpR** fusion providing a simple means of reporting the response of the **hut** locus to histidine, we applied the fusion strain to the plant environment to see whether it was possible to measure **hutT** gene activity in a more complex system. The **hutT::tnpR** fusion strain was inoculated onto sugar beet seedlings (~10^3 cells per seed), which were allowed to germinate and grow in vermiculite (neither the seed nor the vermiculite was sterilized). The same fusion was also propagated in vermiculite devoid of seedlings and on seedlings that were watered on day 0 and day 10 with 5 ml and 3ml of a 10 mg l^-1 solution of histidine, respectively. A control fusion was also included in which the 2.47 kb **hut** fragment was cloned in the opposite (reverse) orientation with respect to the promoterless recombinase. After 2 weeks, *P. fluorescens* cells were recovered from both the rhizosphere and the shoot, and a minimum of 300 colonies from each treatment were replica-plated to check for resolution of the res1-tet-res1 cassette. The results are shown in Table 2 and provide evidence of low, but detectable, levels of recombinase activity (indicative of **hutT** activation) in both the rhizosphere and phyllosphere. Activation of **hutT** was also detected in plant extract. No resolution of the res1-tet-res1 cassette was detected in the rhizosphere-only (no seedling) control; similarly, no resolution of the res1-tet-res1 cassette was detected in the fusion strain in vermiculite.

### Table 2. Resolution level of the **hutT::tnpR** fusion strain of *P. fluorescens* SBW25 in association with sugar beet

Data are means and standard errors from the combined total of bacteria from between one and three plants, from three independent experiments. Raw data are given in parentheses: total number of Tc^r colonies as a fraction of the total number of colonies counted.

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Percentage of Tc^r c.f.u.</th>
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<tbody>
<tr>
<td>Seed inoculation of <strong>hutT::tnpR</strong> fusion strain</td>
<td></td>
</tr>
<tr>
<td>Control: seed inoculation of <strong>hutT::tnpR</strong>, fusion strain with wrong insertion direction</td>
<td>0.4±0.18 (11/2916)</td>
</tr>
<tr>
<td>Control: seed inoculation of <strong>hutT::tnpR</strong>, fusion strain with addition of histidine (10 μg ml^-1)</td>
<td>1.3±0.08 (12/916)</td>
</tr>
<tr>
<td>Control: <strong>hutT::tnpR</strong> fusion strain in vermiculite</td>
<td>0±0 (0/1841)</td>
</tr>
<tr>
<td>Induction by plant extract</td>
<td>1.22±0.40 (15/1239)</td>
</tr>
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</table>

*ANOVA shows highly significant differences among treatments (P <0.0009).

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*Fig. 3.* Effect of histidine concentration on **hutT** gene transcriptional fusions to the reporter genes of **tnpR** (SBW25TC::p675Km-**hutT**) and **lacZY** (IVET fusion PI122), respectively. Data are means and standard errors of three replicates.

*Fig. 4.* Time-course of histidine induction of the **hutT** gene, as measured via transcriptional fusions to the promoterless reporter genes **tnpR** (SBW25TC::p675Km-**hutT**) and **lacZ** (IVET fusion PI122), represented by solid and discontinuous lines, respectively. The transcriptional activation was determined in M9 broth (▲) and M9 broth containing 500 μg histidine ml^-1 (●). Data are means and standard errors of three replicates.
strain containing the 2.47 kb hut fragment in the reverse orientation. While the level of induction was low, ANOVA shows a highly significant effect ($P < 0.0009$).

Taking advantage of the data presented in Fig. 3, which show the relationship between histidine concentration and resolution of the res1-tet-res1 cassette, it is possible to estimate the concentration of histidine present in the plant environment (as sensed by SBW25): 0.39% resolution in the rhizosphere (Table 2) corresponds to approximately 0.6 µg histidine ml$^{-1}$ (3 µM).

**Ecological significance of histidine for bacterial colonization in planta**

Having demonstrated the availability of histidine in the plant environment, we next asked whether histidine plays a significant role in bacterial colonization in planta. To do this we examined the competitive ability of SBW25ΔhutT relative to the wild-type strain during the course of colonization of sugar beet seedlings.

SBW25ΔhutT was mixed 1:1 with a lacZ-marked wild-type strain (SBW25-lacZ; X.-X. Zhang & P. B. Rainey, unpublished results) and the mixed bacteria were inoculated onto sugar beet seeds. Two weeks after inoculation, the plants were destructively harvested and the ratio of mutant to the wild-type competitor was determined by plating on M9 plates supplemented with X-Gal. As a control for any effect due to the lacZ marker, ancestral wild-type SBW25 was competed in parallel against SBW25-lacZ. The results, presented in Fig. 5, show no significant difference between the fitness of the mutant relative to the competitor (SBW25-lacZ), or between the fitness of the ancestral wild-type SBW25 relative to SBW25-lacZ. In other words, SBW25ΔhutT is not compromised in its ability to colonize sugar beet seedlings.

**DISCUSSION**

Here we have described the development and use of the RIVET strategy in *P. fluorescens* SBW25 to monitor *hut* gene activity *in vitro* and *in planta*. Central to this work was a rhizosphere-inducible locus isolated in a previous study and predicted, on the basis of *in silico* analyses, to be involved in histidine uptake and metabolism and thus to be histidine responsive. This prediction was confirmed by showing that the locus is responsive, at a transcriptional level, to histidine. Moreover, analysis of SBW25ΔhutT confirmed that the locus identified by IVET fusion strain PI122 plays a functional role in histidine utilization: on the basis of its similarity to the HutT transporter of *P. putida* and *Pseudomonas aeruginosa* (Rietsch et al., 2004) it is highly likely to function as a histidine-specific transporter.

Armed with the knowledge that the *hut* locus is histidine inducible, we proceeded to apply the RIVET strategy in *P. fluorescens*. Initial trials using the native pIVET5 plasmid (although with a Km-resistance cassette substituting for the lacZ reporter) showed the system to be too sensitive to be useful. Subsequent analysis of two alternative plasmids containing modified *tnpR* RBSs showed that the activity of *tnpR* when controlled by the *tnpR*mut168 RBS closely matched the transcriptional activity of the *hut* locus. Moreover, the response to histidine (across both dose and time) as reported by *tnpR* closely paralleled the response as reported by lacZ.

To demonstrate the utility of the strategy and to verify previous claims that the *hut* locus is rhizosphere inducible (Rainey, 1999), the recombinase reporter was used to measure the activity of *hutT* in the plant (including rhizosphere) environment. Evidence of low, albeit significant, induction was obtained in the rhizosphere, the phyllosphere and in plant extract. The fact that no induction was detected when the *hutT* fusion strain was propagated in vermiculite alone indicates that the inducing signal(s) is plant-derived.

The dose–response curve (Fig. 3) relating histidine concentration to recombinase activity was used to obtain an approximate estimate of the concentration of histidine in the plant environment (3 µM). Interestingly, this estimate is identical to the concentration of histidine reported in root exudates from tomato seedlings (Simons et al., 1997); however, it is based on the assumption that histidine is the sole inducer of *hutT* in the plant environment. In *P. putida*, urocanate (the first breakdown product of histidine) is also able to induce transcription of *hut* (Hu et al., 1989); however, urocanate is not known to occur in the plant environment and is likely to occur solely within bacterial cells as a consequence of the uptake of histidine from the environment and metabolism *in vivo*.

While our results add to the growing list of successful RIVET applications (Casavant et al., 2002, 2003; Lee et al., 1998, 1999; Camilli & Mekalanos, 1995), it is worth noting that the graded dose–response curve seen in this study,
which mirrors those reported elsewhere, is counter-intuitive. Graded responses are expected when transcriptional activity is reported at a population level via an enzyme, such as β-galactosidase, in which the input signal causes a proportional response in output (transcription and enzyme activity). Recombinase reporters ought, in principle, to generate all-or-nothing (threshold) responses: resolution of the res1-tet-res1 cassette requires a certain minimal threshold concentration of recombinase before cells resolve the Tc-resistance gene. Assuming that all cells experience the same environment (as in a shaken broth culture), then resolution should occur in all cells at approximately the same time. The implication is that at the single-cell level, the sensing and response of individual cells to specific signals are highly variable, despite apparent environmental uniformity, a result that we have independently verified using gfp reporter fusions to hut (X.-X. Zhang & P. B. Rainey, unpublished results). Clearly, the response of individual cells to identical levels of inducer is not reproducible at the single-cell level, and suggests a strong probabilistic element to regulatory networks consistent with a number of recent reports (McAdams & Arkin, 1997; Fiering et al., 2000; Leveau & Lindow, 2001; Elowitz et al., 2002). One additional value of recombinase-based reporter strategies may be to provide insights into the balance between stochastic and deterministic processes that control gene regulation.

The inability of the hutT mutant to utilize histidine as a sole carbon and nitrogen source confirms that the biological function of the hut operon is uptake and degradation of histidine. Activation of hutT transcription in the plant environment (in response to plant signals) strongly suggests that histidine is present and available in this environment and can be used as a source of carbon and/or nitrogen by SBW25. The fact that there is no competitive difference between a mutant defective in its ability to utilize an exogenous source of histidine and the wild-type indicates that the ability to utilize histidine in the plant environment does not contribute significantly toward competitive fitness in this environment. This finding is not surprising, given both the oligotrophic nature of the rhizosphere (Poindexter, 1981) and the diverse nutritional capabilities of P. fluorescens (Stanier et al., 1966).

Taken together, our results demonstrate the utility of the recombinase strategy to report, in the plant environment, at the level of the single bacterial cell, transcriptional activity of a single locus in response to specific inducing signals. Further tailoring of the system, possibly via alterations in the translational activity of the recombinase or through modification of the sampling regime (for example, more samples from specific locations along the root) and by studies of the transcriptional activity of additional loci, stands to provide insight into the distribution of inducing signals in complex environments. Such insights into environmental complexity are consistent with those provided by the use of recombinase reporters to study gene expression in V. cholerae in mammalian systems (Lee et al., 1999; Merrell & Camilli, 2000) and, more recently, the availability of arabinose and toluene to Ent. cloacae JL1157 and P. fluorescens AS06 in the plant environment (Casavant et al., 2002, 2003). They also complement green fluorescent protein (GFP)-based work that has demonstrated heterogeneity in the bioavailability of sugar (Leveau & Lindow, 2001) and iron (Joyner & Lindow, 2000), as well as indoleacetic acid metabolism, in epiphytic bacteria (Brandl et al., 2001).

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