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

Xue-Xian Zhang,^{1,2,3} Andrew George,¹ Mark J. Bailey² and Paul B. Rainey^{1,3}

Correspondence

Xue-Xian Zhang
xx.zhang@auckland.ac.nz

¹Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, UK

²Center for Ecology and Hydrology NERC, Mansfield Road, Oxford OX1 3SR, UK

³School of Biological Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand

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 (*tnpR*^{mut168}) 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.

Received 30 November 2005

Revised 11 February 2006

Accepted 22 February 2006

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, ceftriaxone, fucidin and cephalosporin; IVET, *in vivo* expression technology; Km, kanamycin; Pc, piperacillin; 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 'dirty' environment like the rhizosphere can be 'a needle in a haystack' exercise. We note, however, that *gfp* fusions have been successfully used to study the response of individual 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 (*resI*). 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. Recombinase strategies have also been described for the study of toluene and arabinose availability in plant-colonizing *Enterobacter cloacae* JL1157 and *P. fluorescens* A506 (Casavant *et al.*, 2002, 2003). Here we describe the development and application of RIVET to *P. fluorescens* SBW25. Integral to this task was a functional analysis of a previously uncharacterized *rhi* fusion (*rhi14*) (Rainey, 1999) that we show is both histidine inducible and required for histidine utilization. We show that RIVET is able to report, at the single-cell level, the transcriptional activity of the histidine uptake and degradation operon (*hut*) in the plant environment, that low levels of histidine are present in the plant environment and that histidine utilization is not a major determinant of ecological performance *in planta*.

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; piperacillin (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 *resI-tet-resI* 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 *resI-tet-resI* cassette in the middle of a 2.5 kb *KpnI-PstI* fragment located upstream of the well-characterized *wss* operon (Spiers *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 tRNA^{Thr} (Spiers *et al.*, 2002). The *resI-tet-resI* cassette (a 3.6 kb *KpnI-PstI* fragment from pGH436) was cloned into pAS232, which was digested with *HindIII* and blunt-ended 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 homogeneous 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:: *resI-tet-resI*) 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) (*tnpR*^{mut168}, *tnpR*^{mut135}) (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^r 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 *BglII* site of these three plasmids to generate the final constructs p674Km-*hutT*, p675Km-*hutT* and p676Km-*hutT*, which have the *hutT* gene fused to *tnpR*^{wt}, *tnpR*^{mut168} and *tnpR*^{mut135},

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

Strain, plasmid or primer	Relevant characteristics	Source or reference
<i>P. fluorescens</i>		
SBW25	Wild-type strain isolated from phyllosphere of sugar beet	Rainey & Bailey (1996)
SBW25 Δ <i>hutT</i>	<i>hutT</i> deletion mutant of SBW25	X.-X. Zhang & P. B. Rainey, unpublished results
SBW25- <i>lacZ</i>	SBW25 carrying <i>lacZ</i> in a phage locus	X.-X. Zhang & P. B. Rainey, unpublished results
SBW25Tc	SBW25:: <i>res1-tet-res1</i>	This work
PI122	IVET fusion strain for pIVETP:: <i>hutT</i>	Rainey (1999)
<i>E. coli</i>		
DH5 α λ <i>pir</i>	<i>supE44</i> Δ <i>lacU169</i> <i>hsdR17</i> , <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i> , λ <i>pir</i>	Hanahan (1983)
Plasmid		
pRK2013	Helper plasmid, Tra ⁺ , Km ^r	Ditta <i>et al.</i> (1980)
pUIC3	Universal IVET vector with ' <i>lacZ</i> , Mob ⁺ , Tc ^r	Rainey (1999)
pIVETP	IVET vector with ' <i>panB</i> ' <i>lacZ</i> , <i>oriR6K</i> , Mob ⁺ , Tra ⁻ , Pc ^r , Tc ^r	Rainey (1999)
pIVETP1	Derivative of pIVETP without the Tc ^r gene	This work
pUIC3 Δ <i>hutT</i>	pUIC3 containing <i>hutT</i> deletion fragment, Tc ^r	This work
pGH436	pBR322:: <i>res1-tet-res1</i> , Ap ^r , Tc ^r	Stark <i>et al.</i> (1991)
pAS232	pBluescript containing 2.5 kb <i>KpnI</i> – <i>PstI</i> fragment from SBW25	Spiers <i>et al.</i> (2002)
pIVET-Tc4	pIVETP1 carrying the <i>res1-tet-res1</i> cassette in the middle of 2.5 kb fragment from pAS232	This work
pIVET5	RIVET vector with ' <i>tnpR</i> ^{wt} ' <i>lacZY</i> , <i>oriR6K</i> , Mob ⁺ , Tra ⁻ , Ap ^r	Camilli & Mekalanos (1995)
p674Km	Derivative of pIVET5, <i>lacZY</i> was replaced with Km ^r gene	This work
p675Km	Derivative of pIVET5 with <i>tnpR</i> ^{mut168} , <i>lacZY</i> was replaced with Km ^r gene	This work
p676Km	Derivative of pIVET5 with <i>tnpR</i> ^{mut135} , <i>lacZY</i> was replaced with Km ^r gene	This work
p674Km- <i>hutT</i>	p674Km carrying 2.47 kb <i>hut</i> fragment, <i>hutT</i> :: <i>tnpR</i> ^{wt} fusion	This work
p675Km- <i>hutT</i>	p675Km carrying 2.47 kb <i>hut</i> fragment, <i>hutT</i> :: <i>tnpR</i> ^{mut168} fusion	This work
p676Km- <i>hutT</i>	p676Km carrying 2.47 kb <i>hut</i> fragment, <i>hutT</i> :: <i>tnpR</i> ^{mut135} fusion	This work
Primers*		
4KF	5'-GGGGTACCCCTTTCCCAGTCACGACGTTGT-3'	This work
4KR	5'-GGGGTACCCACAGGAAACAGCTATGACCA-3'	This work
PhutT-1F	5'-GAAGATCTCGCTGATGATGGACAAG-3'	
PhutT-1R	5'-cagcatcgatcggtgacggaGACCGGGTTGTGCACGGCCAT-3'	This work
PhutT-2F	5'-tccgtcaacgatcgatgctgGGTGCCGTGTGGATCGTG-3'	This work
PhutT-2R	5'-GAAGATCTGTAACGCGCCGCGAGGCTG-3'	This work

*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* SBW25Tc 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 SBW25 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 *Bgl*II restriction site to generate

pUIC3 Δ *hutT*. To construct the *hutT* deletion mutant of SBW25, plasmid pUIC3 Δ *hutT* was mobilized into *P. fluorescens* SBW25 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 μ g 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^s 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-methylumbelliferyl- β -D-galactoside (4MUG) as the enzymic substrate. The product (7-hydroxy-4-methylcoumarin,

4MU) was detected in a Hoefer DyNA 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 aM 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. Tc^s 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 10³ 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 Tc^s 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 competition were physiologically equal, both the mutant (SBW25Δ*hutT*) 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 *rhi* 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 111th 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

Fig. 1. Transcriptional response of the IVET fusion PI122 to amino acids. β-Galactosidase activities were measured for cells growing in minimal medium M9 with the addition of the indicated individual amino acids at 15.5 µg ml⁻¹. Data are means and standard errors of six replicates.

Fig. 2. Genetic organization of the histidine utilization (*hut*) locus of *P. fluorescens* SBW25 based on the *P. fluorescens* SBW25 genome sequence (<http://www.sanger.ac.uk>). The metabolic genes are shown in empty arrow bars and the putative transporters are shown in grey arrow bars. The *hut* genes are regulated by genes encoding HutC(D) that are represented by black arrow bars. The 2.47 kb insert of the original IVET fusion (PI122) and its orientation are indicated by an arrowed line (accession no. AJ421809). It spanned the last 550 nt of *hutH1*, all of *hutH2*, and through the first 111 nt of *hutT*. The start code of *hutU* and the RBS sequence are shown in bold italic and bold underlined type, respectively. The putative HutC repressor binding site (Hu *et al.*, 1989) is underlined and the predicted δ^{54} -specific promoter sequence is indicated by bold type.

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, SBW25 Δ *hutT* 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 Tn $\gamma\delta$; (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 Tc^s 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 Tc^r 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 μ g 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 Tc^s colonies. The majority of colonies (>90%) from populations grown in the presence of the histidine inducer were Tc^s. However, a similar proportion of cells grown in the absence of the inducer were also Tc^s. 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: *tnpR*^{mut168} and *tnpR*^{mut135}. In *V. cholerae*, both *tnpR*^{mut168} and *tnpR*^{mut135} cause a reduction in the translational efficiency of *tnpR* (Lee *et al.*, 1999). Into both *tnpR*^{mut168} and *tnpR*^{mut135} 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 μ g histidine ml⁻¹ and the ratio of Tc^s to Tc^r was determined by replica-plating. In the presence of histidine, ~45% of cells carrying the *hutT::tnpR*^{mut168} fusion were Tc^s, whereas all colonies carrying the *hutT::tnpR*^{mut135} fusion retained their Tc^r phenotype. No Tc^s cells were detected in the absence of histidine.

The utility of *hutT::tnpR*^{mut168} 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 μ g 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.*

Fig. 3. Effect of histidine concentration on *hutT* gene transcriptional fusions to the reporter genes of *tnpR*^{mut168} (SBW25TC::p675Km-*hutT*) and *lacZY* (IVET fusion PI122), respectively. Data are means and standard errors of three replicates.

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*^{mut168} 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*^{mut168} fusion strain was inoculated onto sugar beet seedlings (~10³ 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 µg ml⁻¹ solution

Fig. 4. Time-course of histidine induction of the *hutT* gene, as measured via transcriptional fusions to the promoterless reporter genes *tnpR*^{mut168} (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.

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 phyllo-sphere. Activation of *hutT* was also detected in plant extract. No resolution of the *res1-tet-res1* cassette was detected in the vermiculite-only (no seedling) control; similarly, no resolution of the *res1-tet-res1* cassette was detected in the fusion

Table 2. Resolution level of the *hutT*::*tnpR*^{mut168} 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^s colonies as a fraction of the total number of colonies counted.

Treatment*	Percentage of Tc ^s c.f.u.	
	Shoot	Rhizosphere
Seed inoculation of <i>hutT</i> :: <i>tnpR</i> ^{mut168} fusion strain	0.4 ± 0.18 (11/2916)	0.39 ± 0.17 (12/2785)
Control: seed inoculation of <i>hutT</i> :: <i>tnpR</i> ^{mut168} , fusion strain with wrong insertion direction	0 ± 0 (0/2146)	0 ± 0 (0/3104)
Control: seed inoculation of <i>hutT</i> :: <i>tnpR</i> ^{mut168} , fusion strain with addition of histidine (10 µg ml ⁻¹)	1.32 ± 0.08 (12/916)	4.68 ± 2.41 (52/1324)
Control: <i>hutT</i> :: <i>tnpR</i> ^{mut168} fusion strain in vermiculite	0 ± 0 (0/1841)	0 ± 0 (2360)
Induction by plant extract	1.22 ± 0.40 (15/1239)	—

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

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 $0.6 \mu\text{g histidine ml}^{-1}$ ($3 \mu\text{M}$).

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 SBW25 Δ *hutT* relative to the wild-type strain during the course of colonization of sugar beet seedlings.

SBW25 Δ *hutT* 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, SBW25 Δ *hutT* is not compromised in its ability to colonize sugar beet seedlings.

Fig. 5. Fitness of *P. fluorescens* SBW25 Δ *hutT* and ancestral *P. fluorescens* SBW25 (wild-type) relative to a *lacZ*-marked strain of *P. fluorescens* SBW25-*lacZ*. White and black bars refer to fitness in the shoot and rhizosphere, respectively. Data are means and standard errors of 10 plants inoculated with independent cultures. ANOVA analysis showed no significant difference among means.

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 SBW25 Δ *hutT* 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 ($3 \mu\text{M}$). 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* A506 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).

ACKNOWLEDGEMENTS

We thank A. Camilli, Tufts University School of Medicine, Boston, MA, for providing the original RIVET plasmids used in this study. Preliminary sequence data used for the *hut* locus were obtained from the *P. fluorescens* SBW25 Sequencing Group at the Sanger Institute (<http://www.sanger.ac.uk>). This work was supported by NERC (UK).

REFERENCES

- Brandl, M. T., Quinones, B. & Lindow, S. E. (2001). Heterogeneous transcription of an indoleacetic acid biosynthetic gene in *Erwinia herbicola* on plant surfaces. *Proc Natl Acad Sci U S A* **98**, 3435–3459.
- Camilli, A. & Mekalanos, J. J. (1995). Use of recombinase gene fusions to identify *Vibrio cholerae* genes induced during infection. *Mol Microbiol* **18**, 671–683.
- Camilli, A., Beattie, D. T. & Mekalanos, J. J. (1994). Use of genetic recombination as a reporter of gene expression. *Proc Natl Acad Sci U S A* **91**, 2634–2638.
- Casavant, N. C., Beattie, G. A., Phillips, G. J. & Halverson, L. J. (2002). Site-specific recombination-based genetic system for reporting transient or low-level gene expression. *Appl Environ Microbiol* **68**, 3588–3596.
- Casavant, N. C., Thompson, D., Beattie, G. A., Phillips, G. J. & Halverson, L. J. (2003). Use of a site-specific recombination-based biosensor for detecting bioavailable toluene and related compounds on roots. *Environ Microbiol* **5**, 238–249.
- Ditta, G., Stanfield, S., Corbin, D. & Helinski, D. R. (1980). Broad host range DNA cloning system for Gram negative bacteria: construction of a gene bank of *Rhizobium meliloti*. *Proc Natl Acad Sci U S A* **77**, 7347–7351.
- Elowitz, M. B., Levine, A. J., Siggia, E. D. & Swain, P. S. (2002). Stochastic gene expression in a single cell. *Science* **297**, 1183–1186.
- Fiering, S., Whitelaw, E. & Martin, D. I. K. (2000). To be or not to be active: the stochastic nature of enhancer action. *BioEssays* **22**, 381–387.
- Gal, M., Preston, G. M., Massey, R. C., Spiers, A. J. & Rainey, P. B. (2003). Genes encoding a cellulose polymer contribute toward the ecological success of *Pseudomonas fluorescens* SBW25 on plant surfaces. *Mol Ecol* **12**, 3109–3121.
- Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–580.
- Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K. & Pease, L. R. (1989). Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* **77**, 61–68.
- Hu, L., Allison, S. L. & Phillips, A. T. (1989). Identification of multiple repressor recognition sites in the *hut* system of *Pseudomonas putida*. *J Bacteriol* **171**, 4189–4195.
- Joyner, D. C. & Lindow, S. E. (2000). Heterogeneity of iron bioavailability on plants assessed with a whole-cell GFP-based bacterial biosensor. *Microbiology* **146**, 2435–2445.
- Lee, S. H., Angelichio, M. J., Mekalanos, J. J. & Camilli, A. (1998). Nucleotide sequence and spatiotemporal expression of the

- Vibrio cholerae* *vieSAB* genes during infection. *J Bacteriol* **180**, 2298–2305.
- Lee, S. H., Hava, D. L., Waldor, M. K. & Camilli, A. (1999). Regulation and temporal expression patterns of *Vibrio cholerae* virulence genes during infection. *Cell* **99**, 625–634.
- Lenski, R. E. (1991). Quantifying fitness and gene stability in microorganisms. *Biotechnology* **15**, 173–192.
- Leveau, J. H. J. & Lindow, S. E. (2001). Appetite of an epiphyte: quantitative monitoring of bacterial sugar consumption in the phyllosphere. *Proc Natl Acad Sci U S A* **98**, 3446–3453.
- Lindow, S. E. & Brandl, M. T. (2003). Microbiology of the phyllosphere. *Appl Environ Microbiol* **69**, 1875–1883.
- McAdams, H. H. & Arkin, A. (1997). Stochastic mechanisms in gene expression. *Proc Natl Acad Sci U S A* **94**, 814–819.
- Merrell, D. S. & Camilli, A. (2000). Detection and analysis of gene expression during infection by in vivo expression technology. *Philos Trans R Soc Lond B* **355**, 587–599.
- Miller, J. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Poindexter, J. S. (1981). Oligotrophy. *Adv Microb Ecol* **5**, 63–89.
- Preston, G. M., Bertrand, N. & Rainey, P. B. (2001). Type III secretion in plant growth-promoting *Pseudomonas fluorescens* SBW25. *Mol Microbiol* **41**, 999–1014.
- Rainey, P. B. (1999). Adaptation of *Pseudomonas fluorescens* to the plant rhizosphere. *Environ Microbiol* **1**, 243–257.
- Rainey, P. B. & Bailey, M. J. (1996). Physical and genetic map of the *Pseudomonas fluorescens* SBW25 chromosome. *Mol Microbiol* **19**, 521–533.
- Rainey, P. B. & Preston, G. M. (2000). In vivo expression technology strategies: valuable tools for biotechnology. *Curr Opin Biotechnol* **11**, 440–444.
- Rietsch, A., Wolfgang, M. C. & Mekalanos, J. J. (2004). Effect of metabolic imbalance on expression of type III secretion genes in *Pseudomonas aeruginosa*. *Infect Immun* **72**, 1383–1390.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Simons, M., Permentier, H. P., de Weger, L. A., Wijffelman, C. A. & Lugtenberg, B. J. J. (1997). Amino acid synthesis is necessary for tomato root colonization by *Pseudomonas fluorescens* strain WCS365. *Mol Plant Microbe Interact* **10**, 102–106.
- Spiers, A. J., Kahn, S. G., Bohannon, J., Travisano, M. & Rainey, P. B. (2002). Adaptive divergence in experimental populations of *Pseudomonas fluorescens*. I. Genetic and phenotypic bases of wrinkly spreader fitness. *Genetics* **161**, 33–46.
- Spiers, A. J., Bohannon, J., Gerhrig, S. M. & Rainey, P. B. (2003). Biofilm formation at the air-liquid interface by the *Pseudomonas fluorescens* SBW25 wrinkly spreader requires an acetylated form of cellulose. *Mol Microbiol* **50**, 15–27.
- Stanier, R. Y., Palleroni, N. J. & Doudoroff, M. (1966). The aerobic pseudomonads: a taxonomic study. *J Gen Microbiol* **43**, 159–271.
- Stark, W. M., Grindley, N. D. F., Hatfull, G. F. & Boocock, M. R. (1991). Resolvase-catalysed reactions between *res* sites differing in the central dinucleotide of subsite. *EMBO J* **10**, 3541–3548.
- Zhang, X.-X., Lilley, A. K., Bailey, M. J. & Rainey, P. B. (2004a). The indigenous *Pseudomonas* plasmid pQBR103 encodes plant-inducible genes including three putative helicases. *FEMS Microbiol Ecol* **51**, 9–17.
- Zhang, X.-X., Lilley, A. K., Bailey, M. J. & Rainey, P. B. (2004b). Functional and phylogenetic analysis of a plant-inducible oligoribonuclease (*orn*) gene from an indigenous *Pseudomonas* plasmid. *Microbiology* **150**, 2889–2898.