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Adaptation of *Pseudomonas fluorescens* to the plant rhizosphere

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Summary

Saprophytic *Pseudomonas* are common root-colonizing bacteria that can improve plant health. Efficient exploitation of these bacteria in agriculture requires knowledge of traits that enhance ecological performance in the rhizosphere. Here, I describe the development and application of a promoter-trapping technology (IVET) that enables the isolation of *Pseudomonas fluorescens* genes that show elevated levels of expression in the rhizosphere. Using IVET, 20 *P. fluorescens* genes were identified that are induced during rhizosphere colonization, and their patterns of expression were analysed in laboratory media and in the rhizosphere. Fourteen genes showed significant homology to sequences in GenBank that are involved in nutrient acquisition, stress response, or secretion; six showed no homology. Seven of the rhizosphere-induced (*rhi*) genes have homology to known non-*Pseudomonas* genes. One of the *rhi* genes (*hrcC*) is a component of a type III secretion pathway, not previously known in non-parasitic bacteria. Together, these genes provide a view of the rhizosphere environment as perceived by a rhizosphere colonist, and suggest that the nature of the association between *P. fluorescens* and the plant root may be more complex and intimate than previously thought.

Introduction

The region of soil surrounding and including the plant root (the rhizosphere) is of crucial importance for plant health and nutrition (Marschner, 1995). The rhizosphere harbours a large and diverse community of prokaryotic and eukaryotic microbes that interact and compete with each other and with the plant root. Activity of any one member of this community affects the growth and the physiology of the others, and also affects the physical and chemical properties of the soil. Together, these interactions set up gradients along individual roots for mineral nutrients, pH, redox potential, reducing processes, root exudates and microbial processes. The totality of these interactions and gradients affects not only mineral nutrient uptake but also the adaptation of plants to adverse soil chemical conditions and susceptibility to disease. Understanding the complexity of this environment and how the microbial community adapts and responds to alterations in the physical, chemical and biological properties of the rhizosphere remains a significant challenge for plant and microbial biologists (Handelsman and Stabb, 1996).

Pseudomonas is an important component of the rhizosphere, and certain isolates can enhance plant health (Schippers *et al.*, 1987; Weller, 1988; Cook *et al.*, 1995). Plant growth-promoting *Pseudomonas* species (collectively known as plant growth-promoting rhizobacteria, PGPRs) exert their beneficial effect via several different mechanisms but principally by active exclusion of pathogens from the rhizosphere. Traits with a confirmed role in this process include allelopathic factors, such as toxins, antibiotics and siderophores (for a review, see Handelsman and Stabb, 1996; Thomashow and Weller, 1996). Recent reports also indicate that some PGPRs can protect plants against pathogen infection by eliciting induction of systemic resistance (ISR) (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997), although the mechanism by which the response is triggered is unclear. Despite obvious benefits for agriculture, attempts to exploit PGPRs as biocontrol inoculants, biofertilizers, phytostimulants or inoculants for combined bacteria–plant bioremediation have met with limited success. Small-scale trials often show spectacular promise, but results from field experiments are typically inconsistent (Handelsman and Stabb, 1996). A major factor contributing to these inconsistent results is variable ecological performance (Weller, 1988).

The ecological performance of rhizosphere-colonizing bacteria (their fitness) is a complex phenotype affected by many different traits and by environmental factors. To date, characters identified include motility (de Weger *et al.*, 1987), synthesis of the O-antigen of lipopolysaccharide (de Weger *et al.*, 1989) and cellulose (Matthysse and McMahan, 1998), thiamine production (Simons *et al.*, 1996), amino acid synthesis (Simons *et al.*, 1997), biotin production (Streit *et al.*, 1996), and an isoflavanoid-inducible efflux pump (Paumbo *et al.*, 1998). More recently, a two-component system (Dekkers *et al.*, 1998a) and a site-specific recombinase

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(Dekkers *et al*., 1998b) have also been discovered, however the function and ecological role of these are unknown.

Ecological performance can be studied by several different methods (Dawkins, 1982; Dykhuizen, 1995; Lenski, 1995). Bottom-up (genes to population) and top-down (population to genes) approaches are both useful. The bottom-up approach is commonly used for studies of bacteria, although it is rarely pursued to the population level. The typical genes-to-phenotype strategy involves identification of traits on the basis of gene inactivation. This is a powerful approach that has been fundamental to the majority of advances in molecular microbiology, but, despite its power, insertional mutagenesis is not always appropriate for the analysis of phenotypes as complex as ecological performance. For most organisms, in most environments, there is no primary determinant of ecological performance; this is because it is determined by complex epistatic interactions among many different gene products that each has a long evolutionary history. Traits having the greatest effect on ecological performance are likely to be those that show subtle quantitative variation, and such traits are unlikely to produce 'defective' phenotypes when inactivated (Lenski, 1995).

Recent advances in gene fusion technologies provide an alternative way to study complex phenotypes. Rather than identifying genes on the basis of function loss, ecologically significant genes can be identified on the basis of their positive contribution towards a specific phenotype. A study with the aim of understanding the mechanistic basis of ecological performance in a PGPR might, therefore, begin by identifying those genes that are specifically induced in the rhizosphere. One advantage of this approach is that it considers bacteria as integrated organisms rather than as a toolbox of independent genes and phenotypes. Ultimately, the bottom-up, positive selection approaches open the door to understanding fitness in bacteria, at a mechanistic level, in the wild.

The first gene fusion technology involving positive selection was developed to study pathogenicity in *Xanthomonas campestris* on turnip seedlings (Osbourn *et al*., 1987). The strategy involved selection of environment-specific genes on the basis of their ability to drive expression of a gene that is essential for survival. In 1993, this strategy was modified and extended to the study of *Salmonella* pathogenicity in a mammalian host, in which the term *in vivo* expression technology (IVET) was coined (Mahan *et al*., 1993). IVET has since been applied extensively to the study of *Salmonella* virulence and has resulted in the discovery of many novel genes; it has also provided valuable insights into the ecology of the host environment (Mahan *et al*., 1995; Heithoff *et al*., 1997; Conner *et al*., 1998). Recently, IVET strategies have been developed for several other bacterial pathogens, including *Vibrio cholerae* (Camilli and Mekalanos, 1995), *Pseudomonas aeruginosa* (Wang *et al*., 1996), *Yersinia enterocolitica* (Young and Miller, 1997) and *Staphylococcus aureus* (Lowe *et al*., 1998).

This paper describes the first step in a long-term project aimed at determining the phenotypic and genetic causes of ecological performance in a rhizosphere-colonizing isolate of *P. fluorescens* (SBW25). *P. fluorescens* SBW25 is a common saprophytic pseudomonad that is able to survive and persist on plant surfaces for extended periods (De Leij *et al*., 1995; Thompson *et al*., 1995). It also protects sugar beet seedlings against damping-off disease caused by *Pythium ultimum* (Ellis, 1998; P. B. Rainey and M. J. Bailey, unpublished). In addition, its genome has been mapped (Rainey and Bailey, 1996); it is used in studies of plasmid transfer in the wild (Lilley and Bailey, 1997) and is the focus of work on the evolution of niche specialization (Rainey and Travisano, 1998). Our approach to understanding the mechanistic bases of rhizosphere colonization in SBW25 begins with the IVET strategy; the development and application of which are described here.

Results

The selection strategy developed for *P. fluorescens* is analogous to IVET developed for *Salmonella* (Mahan *et al*., 1993), but is based upon random integration of promoterless *panB* into the chromosome of a *P. fluorescens* strain carrying a chromosomal deletion of *panB*. The general strategy is outlined in Fig. 1.

Construction and characterization of pantothenate-requiring mutants of P. fluorescens

The power of any IVET strategy is dependent upon the strength of *in situ* selection. The nutritional status of the rhizosphere is not well understood, and few mutants defective in their ability to grow in this environment have been described. To identify conditional lethal mutants, a range of mini-Tn*5*-generated auxotrophs of *P. fluorescens* SBW25 were screened by competitive assay for their inability to colonize the rhizosphere of sugar beet seedlings. A number of useful mutants were obtained (to be reported fully elsewhere), and one of these, PF126 — a pantothenate-requiring auxotroph, formed the basis of the IVET strategy described here.

Pantothenate is a water-soluble B-group vitamin essential for the biosynthesis of coenzyme A (CoA) and acyl carrier protein (ACP). It is also a cofactor in more that 100 reactions in intermediary metabolism (Jackowski, 1996). Figure 2 shows that PF126 is significantly impaired in its ability to colonize the rhizosphere. The large error bars on days 14 and 21 reflect the fact that in some replicates the mutant became extinct. An exogenous source of

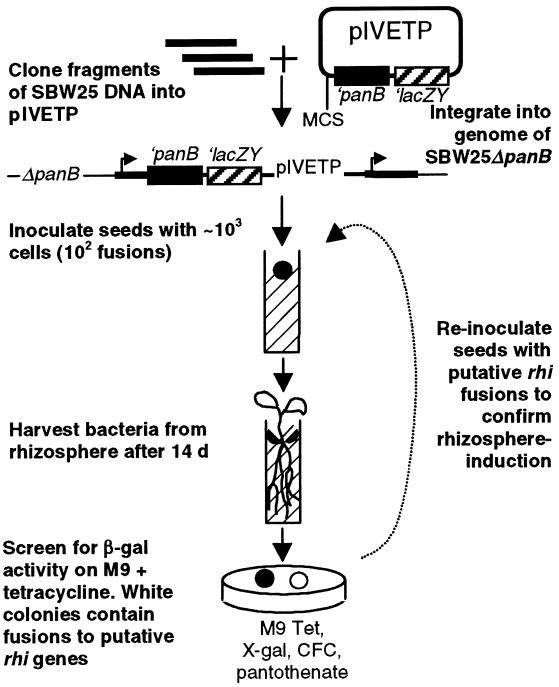

Fig. 1. Selection for genes showing rhizosphere-specific elevation in expression. Genes displaying elevated levels of expression in the rhizosphere are selected on the basis of their ability to drive the expression of a gene that is essential for survival in the rhizosphere. The strategy is based upon random integration of promoterless *panB* (which encodes an enzyme essential for pantothenate biosynthesis) into the chromosome of *P. fluorescens* SBW25 strain carrying a chromosomal deletion of *panB*. SBW25Δ*panB* cannot colonize the rhizosphere because pantothenate is absent or severely limiting, therefore colonization can only occur if the promoterless *panB* gene is inserted downstream of an active promoter. Recovery of strains from the rhizosphere after selection for the ability to synthesize pantothenate results in the isolation of promoters that are either constitutive or rhizosphere specific. To distinguish between these, a promoterless marker operon, *lacZY*, is fused to *panB*, enabling the lactose phenotype of the recovered cells to be determined. Lac^+ strains contain fusions to constitutive promoters (dark colonies), whereas Lac^- strains (white colonies) contain fusions to promoters activated in response to rhizosphere signals.

pantothenate applied to the seed at the time of inoculation rescued the mutant, and therefore the poor colonization ability of PF126 could be attributed solely to the scarcity of pantothenate from the rhizosphere environment.

To determine the genotype of PF126, DNA complementing the pantothenate lesion was obtained from a SBW25 genomic cosmid library, subcloned and sequenced. The DNA sequence from the smallest clone capable of restoring PF126 to prototrophy, pPS126-18 (Fig. 3A), revealed the presence of *panB*, flanked by *folK* and *panC* (accession number AJ130846). The *panB* gene encodes a protein of 267 amino acids that is 54% identical to PanB from *Escherichia coli*. Confirmation of functional identity was obtained by showing that *panB* from SBW25 was able to restore prototrophy on the *E. coli panB* mutant

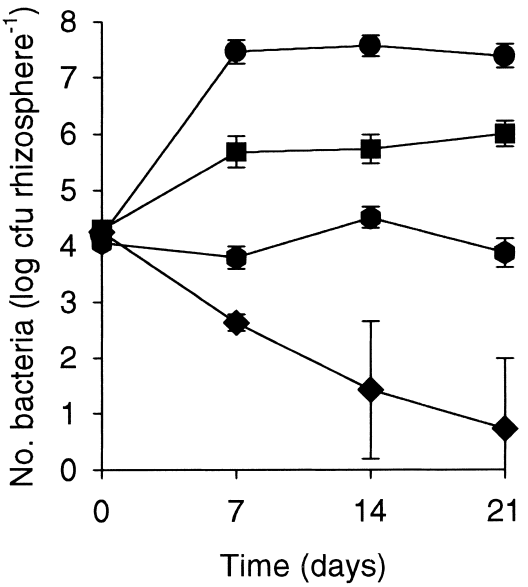

Fig. 2. Colonization of the sugar beet rhizosphere by pantothenate-requiring mutants of *P. fluorescens* SBW25. *P. fluorescens* SBW25 (●), PF126 (♦), PF126(pPS126) (■), SBW25Δ*panB* (⬢). Data points are the means and standard errors from experiments carried out in triplicate. The difference between PF126(pPS126) and SBW25 reflects the cost of carriage of pPS126.

strain YA139 (Cronan, 1980). In *E. coli*, *panB* is the first gene of the pantothenate biosynthetic operon and encodes ketopantoate hydroxymethyltransferase, which catalyses the formation of ketopantoate from α-ketoisovalerate and

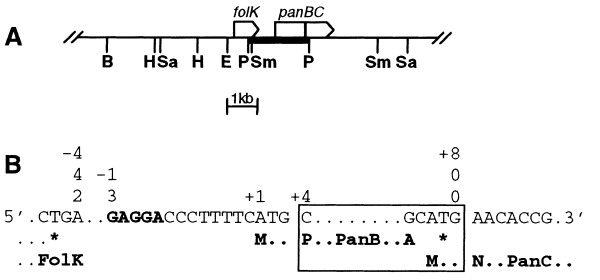

Fig. 3. Genetic organization of the pantothenate operon and detail of SBW25Δ*panB*.
A. Restriction map of cosmid pPS126 showing *folK*, and the first two genes of the pantothenate biosynthetic operon, *panBC*. The dark line denotes the 1.8 kb *Pst*I fragment that was the starting point for construction of the *panB* deletion (accession number AJ130846). Direction of transcription is indicated by the arrows.
B. Detail of the nucleotide (and amino acid) sequence of the SBW25 chromosome surrounding the *panB* gene. *folK* ends 441 nucleotides from the ATG start (+1) of *panB*. *panC* is translationally coupled to *panB* and requires either a −1 or +2 frameshift for correct translation. The 797 nucleotides of *panB* within the boxed region (+4 to +800) were deleted from the chromosome to generate SBW25Δ*panB*. The putative ribosome binding site (RBS) is shown in bold. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Sa, *Sal*I; Sm, *Sma*I.

is the first committed step in the biosynthesis of pantothenate (Teller *et al*., 1976).

To circumvent a range of potential problems associated with the use of a transposon mutant as the basis of the IVET selection strategy, a *panB* deletion mutant of SBW25 was generated. This was achieved by deleting the entire *panB* ORF from the chromosome of SBW25 using a two-step allelic exchange strategy (see *Experimental procedures*). The deletion was designed to ensure that the entire *panB* sequence was removed, thus fusing the *panB* start codon with the second codon of *panC* (Fig. 3B). Unlike the transposon mutant, the *panB* deletion had no polar effects; furthermore, the possibility of recombination between the IVET vector (which contains the *panB* sequence; see below) and the chromosome was eliminated. SBW25Δ*panB* was unable to colonize the rhizosphere (Fig. 2).

Construction of IVET selection strategy for P. fluorescens

Plasmid pIVETP was constructed to allow transcriptional fusions of random *P. fluorescens* DNA fragments to a promoterless *panB–lacZY* cassette. Figure 4A shows the universal IVET vector (pUIC3) into which a promoterless copy of the SBW25 *panB* gene plus a ribosome binding site was cloned to generate pIVETP (Fig. 4B). The completed vector has several important features. First, it contains a unique *Bgl*II site into which partially *Sau*3AI-digested *P. fluorescens* DNA can be cloned. Second, when introduced into *P. fluorescens* SBW25Δ*panB*, the vector integrates into the genome by a single homologous recombination event that is readily selected by screening for tetracycline resistance. Third, the presence of translational stop codons in the multiple cloning site (MCS) prevents translational fusions between cloned DNA fragments and *panB*; and, fourth, the presence of *Pac*I and *Spe*I sites means that the fusion can be readily positioned on the SBW25 genome map (Rainey and Bailey, 1996).

Isolation of P. fluorescens *genes induced in the sugar beet rhizosphere*

To enrich for *P. fluorescens* genes displaying elevated levels of expression in the rhizosphere, a *P. fluorescens* chromosomal fusion library was constructed in pIVETP, and the library was mobilized from *E. coli* into SBW25Δ*panB* by conjugation. Chromosomally integrated fusions were selected by plating on medium containing tetracycline. Selection for rhizosphere-induced genes (*rhi* genes) occurred after inoculation of pools of fusion strains onto seeds that were germinated and grown for 14 days (Fig. 1).

From ≈5000 IVET-selected colonies plated, 40 Lac$^-$ colonies were selected for further investigation. Chromosomally integrated *rhi* fusions were recovered from the SBW25Δ*panB* genome by conjugative cloning and were restriction mapped to eliminate siblings. Twenty fusion joint points were sequenced, and Table 1 shows the similarity to DNA and protein sequences contained in GenBank/EMBL and SWISSPROT databases and the unfinished *P. aeruginosa* genome sequence.

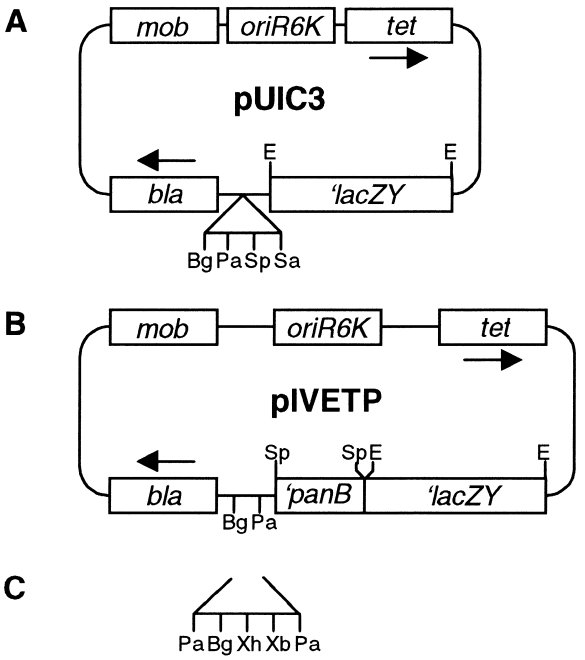

Fig. 4. Construction of pIVETP.
A. pUIC3 is an intermediate in the construction of *Pseudomonas* IVET vectors. It is based upon pGP704 and includes a tetracycline resistance gene, promoterless *lacZY* operon [including ribosome binding site (RBS)] and multiple cloning site (MCS). Immediately downstream of the unique *Bgl*II site in the MCS are translational stop codons in the three forward reading frames. The *Spe*I site provides a convenient position for insertion of rhizosphere-selected markers. The promoterless *lacZY* operon can be readily excised and replaced with alternative markers.
B. pIVETP is pUIC3 with the promoterless *panB* gene from *P. fluorescens* cloned into the *Spe*I site.
C. A modification to the MCS of pIVETP, which contains two sites for library construction (*Bgl*II and *Xho*I) flanked by rare cutting sites. Bg, *Bgl*II; E, *Eco*RI; Pa, *Pac*I; Sa, *Sal*I; Sp, *Spe*I; Xb, *Xba*I; Xh, *Xho*I.

Induction of rhi *genes is necessary for colonization of the rhizosphere*

All IVET-selected *rhi* genes were Lac$^-$ and auxotrophic on M9 minimal medium, but could be rescued by the addition of pantothenate. As pantothenate-requiring auxotrophs of SBW25 are unable to colonize the rhizosphere, *rhi* genes must be induced in the rhizosphere to ensure transcription of *panB* and thus growth of the fusion strains. To confirm that *rhi* genes are induced in the rhizosphere, the ability

Table 1. Predicted function of *rhi* loci.

Fusion	Locus	Amino acid identity, protein, organism[a]	Predicted function or property	*P. aeruginosa* homologue?[b]
		Nutrient acquisition		
PI009	*rhi-2*	38%, *dctS, R. capsulatus* (X64733)	C4-dicarboxylate transport sensor protein	No homologue
PI142	*rhi-17*	50%, *xylA, H. influenzae* (U32791)	Xylose metabolism	No homologue
PI012	*rhi-4*	41%, *morB, P. putida* (U37350)	Reductase; degradation of complex N compounds	67%
PI117	*rhi-10*	62%, *livMH, E. coli* (P22729)[c]	High-affinity branched chain amino acid transport permease	64%
PI122	*rhi-14*	96%, *hutT, P. putida* (AF032970)	Inducible histidine transporter	87%
		Secretion		
PI121	*rhi-15*	48%, *tig, E. coli* (U82664)	Trigger factor	67% §
PI143	*rhi-18*	53%, *hrcC, E. amylovora* (U56662)[d]	Component of type III secretion system	22% §
PI055	*rhi-8*	51%, *ragC, B. japonicum* (AJ225023)	Cation efflux system	No homologue
PI148	*rhi-19*	63%, *rosA, Y. enterocolitica* (U46859)	Transmembrane protein fosmidomycin resistance	No homologue
		Stress response		
PI003	*rhi-1*	24%, *dnrO, S. peucetius* (L37338)	Putative repressor	No homologue
PI011	*rhi-3*	75%, *copRS, P. syringae* (L05176)[e]	Copper-inducible regulator	84%†
PI014	*rhi-5*	53%, *ykmA, B. subtillis* (Z99110)	Glutathione peroxidase: oxidative stress response	68%† 58%
PI162	*rhi-21*	39%, *ycbL, H. influenzae* (Q57544)	Member of glyoxylase II family of enzymes	84%
PI125	*rhi-12*	91%, *lsfA, P. putida* (AF075709)[f]	Oxidative stress response	88%
		Other non-categorized functions		
PI021	*rhi-6*	None detected[g]		No homologue
PI043	*rhi-7*	None detected		78%
PI111	*rhi-9*	None detected		No homologue
PI119	*rhi-11*	None detected		No homologue
PI127	*rhi-13*	None detected		No homologue
Pi157	*rhi-20*	None detected		No homologue

a. Bacterial proteins with greatest amino acid identity to predicted *rhi* proteins. SWISSPROT or GenBank database accession numbers are shown in parentheses.
b. Result of TBLASTX search against the uncompleted *P. aeruginosa* genome sequence (www.pseudomonas.com): % identity at amino acid level; fusions with same symbol are located on the same contig.
c. Fusion spans junction between *iivM* and *iivH*. Amino acid identity to LivH (permease) is 59% (P08340). *P. aeruginosa* has a homologue that is 84% identical and located on the same contig as *rhi-10*.
d. DNA sequence of the fusion from the *bla* gene revealed a putative gene that is 63% identical to *hrcJ* from *P. syringae* pv. *syringae* (U25813). *P. aeruginosa* has a homologue that is 45% identical and located on the same contig as *rhi-18*.
e. Fusion spans the junction between *copR* and *copS*. Amino acid identity to CopS (copper-inducible sensor) is 31% (L05176).
f. This gene is in the wrong orientation for transcription of *panB*. There are 330 nucleotides between the start codon of *IsfA* and promoterless *panB* (see text). DNA sequence of the fusion from the *bla* gene revealed a putative gene that is 61% identical to a sulphonate-binding protein precursor induced in response to sulphate starvation (AF075709). *P. aeruginosa* has a homologue that is 80% identical and located on the same contig as *rhi-12*.
g. No significant matches to DNA or protein databases.

of each *rhi* fusion strain to colonize the rhizosphere was determined by triplicate assay. Within 14 days of inoculation, all *rhi* fusion strains were present in the rhizosphere at between 4×10^7 and 8×10^7 bacteria per rhizosphere. This number was not significantly different from either the wild-type control strain (SBW25) or a preselected Lac$^+$ strain, which is shown in Fig. 5. This contrasts markedly with the growth of three randomly chosen Lac$^-$ colonies from libraries not subjected to rhizosphere selection. The number of these preselected Lac$^-$ strains showed little increase during the course of 14 days. These data show that individual *rhi* genes are induced in response to rhizosphere signals and that induction is necessary for colonization of the rhizosphere.

The level of rhi *induction differs between* rhi *fusion strains*

To provide further proof of rhizosphere induction and to determine the magnitude of induction, the β-galactosidase

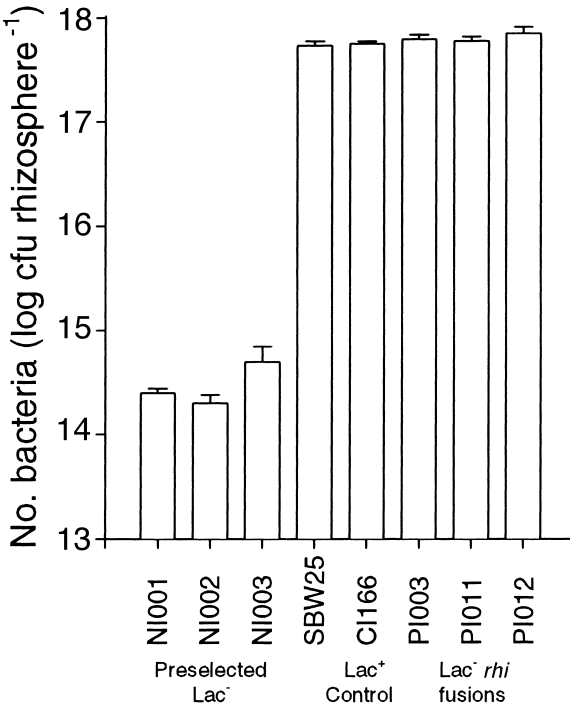

Fig. 5. Induction of *rhi* genes is required for colonization of the rhizosphere. Seeds were inoculated with 10^4 cells of all 20 *rhi* fusion strains, preselected Lac$^-$ strains and a preselected Lac$^+$ fusion strain. All were grown in direct competition with the ancestral strain SBW25-Sm. After 14 days, the 20 *rhi* fusion strains were present at between 4×10^7 and 8×10^7 bacteria per rhizosphere, but only *rhi* fusion strains PI003, PI011, PI012 are shown. Data are means and standard errors from triplicate plants.

activity of *rhi* fusions recovered from the rhizosphere was determined and compared with the β-galactosidase activity of the same strain grown overnight in rich laboratory Luria–Bertani (LB) medium. Figure 6 shows that the levels of induction in cells harvested directly from the rhizosphere were ≈5- to 200-fold greater than the same cells grown in LB.

Comparative analysis of rhi *genes*

Table 1 shows that two sequences were essentially identical to previously known genes from *Pseudomonas putida*, 12 were homologous to known genes from a range of bacteria including other pseudomonads, *E. coli*, *Yersinia enterocolitica* and *Bacillus subtilis*, and six shared no homology with sequences in the DNA database or encode open reading frames with no assigned function. However, one of these did share homology with an uncharacterized region of the *P. aeruginosa* genome. On the basis of their similarity to genes in the DNA databases, the *rhi* genes were subdivided into four categories (Table 1), those with a putative role in nutrient acquisition, stress response and secretion; the fourth contained fusions with no known function.

Two of the fusions with a likely role in nutrient acquisition are predicted to encode polypeptides involved in the uptake of amino acids. Locus *rhi-10* spans the junction between two genes [*livM* (*braE*) and *livH* (*braD*)] that are similar to genes in the LIV-I and LS transport operon of *E. coli* (Adams *et al.*, 1990) and the *braCDEFG* operon of *P. aeruginosa* (Hoshino and Kose, 1990). LivM (BraE) and LivH (BraD), together, form part of a membrane-translocating permease essential for high-affinity transport of branched chain amino acids (Adams *et al.*, 1990). The *braCDEFG* operon of *P. aeruginosa* is highly similar to the *E. coli* transport system, but is less similar to *rhi-10* than the corresponding *E. coli* genes. A search of the uncompleted *P. aeruginosa* genome sequence revealed a contig that spans the junction between two hypothetical ORFs (separated by 11 nucleotides) that share 64% and 84% identity at the amino acid level with the *P. fluorescens* homologues of *livM* and *livH* respectively. This region is not part of the previously characterized *braCDEFG* operon (which is located elsewhere in the genome), and suggests the existence of a second high-affinity amino acid transport pathway in *P. aeruginosa*.

The second locus with a predicted role in amino acid acquisition, *rhi-14*, is highly similar to a conserved family of amino acid transporter permeases. For example, *rhi-14* shares 96% identity to *hutT* from *P. putida* (a histidine-inducible permease), and 93% and 80% identity to *proY* from *Salmonella* and *E. coli* respectively. *proY* is a cryptic proline-specific permease, which unlike the low-affinity proline transporter system allows *Salmonella* to utilize proline as a sole source of both carbon and nitrogen (Liao *et al.*, 1997).

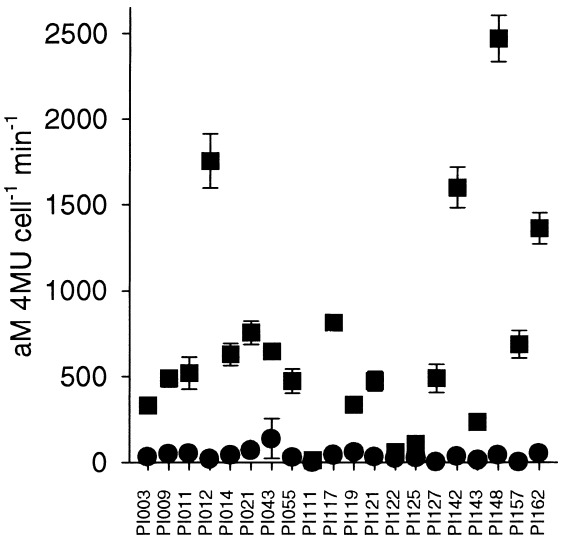

Fig. 6. Induction of *rhi* genes in the sugar beet rhizosphere. β-Galactosidase expression from bacterial cells recovered from the sugar beet rhizosphere (■) compared with the same strain grown in LB (●). Data are means and standard errors from three independent replicates.

Xylose is a typical plant-derived sugar commonly found in plant root exudate that is able to induce expression of the xylose utilization pathway (Song and Park, 1997). Many strains of *Pseudomonas* are able to utilize this compound (Palleroni, 1984), although little is known about the genetic organization of the pathway. The fusion in strain PI142 (*rhi-17*) includes 123 nucleotides from the 3′ end of a gene that encodes a protein that bears strong similarity to *xylA* (xylose isomerase) from a range of bacteria, including *Haemophilus influenzae*, *E. coli* and *Bacillus*. In these bacteria, *xylA* is the first gene of the *xylAB* operon, but no evidence of *xylB* (xylulose kinase) was found in *P. fluorescens*. Instead, between the 3′ end of the *xylA* homologue and the start of the promoterless *panB* gene are 300 nucleotides that show no similarity to any protein sequence in the databases and contain no obvious terminator sequences. However, within this region is a 43 nucleotide motif that is 93% identical to a nucleotide sequence found in *P. fluorescens* DSM50106 (accession number AF007800), which forms part of a 200-nucleotide region that resides between two genes of the mannitol utilization operon, *mltD* (mannitol dehydrogenase) and *mltY* (xylulose kinase) (Brunker *et al*., 1998).

Locus *rhi-4* also has a predicted role in nutrient acquisition. *rhi-4* is 41% identical to both morphinone reductase (*morB*) from *P. putida* M10 (French *et al*., 1995) and NAD(P)H-dependent 2-cyclohexen-1-one reductase (*ncr*) from *Pseudomonas syringae* pv. *glycinea* PG4180.N9 (Rohde *et al*., 1999). It is also similar to GTN reductase from *Agrobacterium radiobacter* (41% identical) (Snape *et al*., 1997) and pentaerythritol tetranitrate (PETN) from *Enterobacter cloacae* (42% identical) (French *et al*., 1998). These enzymes belong to the alpha/beta barrel flavoprotein group of proteins and are similar to old yellow enzyme (NADPH dehydrogenase) from *Saccharomyces cerevisiae*. There is considerable interest in these enzymes because they confer the ability to utilize GTN and related explosives as sources of nitrogen for growth (Snape *et al*., 1997). MorB is also used in production of semisynthetic opiates (French *et al*., 1998). In *P. syringae* pv. *glycinea*, *ncr* was identified after a screen of genes differentially expressed at virulence-promoting (low) temperature (Rohde *et al*., 1999). The precise ecological role of these enzymes is unknown, but it is likely that they are involved in the utilization of complex nitrogen compounds for growth. Interestingly, locus *rhi-4 has* been recovered on three independent occasions, once from the rhizosphere, once from young cotyledons and once from the growing root tip using an IVET strategy based upon stringent selection for diaminopimelic acid production (M. Gal and P. B. Rainey, unpublished). The repeated isolation of this gene suggests a significant role in seedling colonization that is currently being tested through mutational analyses.

Genes involved in the general stress response pathways have been recovered from a number of pathogenic bacteria using IVET. For example, glutathione synthase and glutaredoxin were shown to be involved in *Yersinia enterocolitica* pathogenesis, in which they are thought to play a role in enhancing survival in host tissues. Both *rhi-5* and *rhi-12* identify ORFs with homology to glutathione peroxidases, which are thiol-specific antioxidants that play an important role in protecting cells against oxidative damage. Unfortunately, it is unclear which gene in *rhi-12* is rhizosphere induced because the direction of transcription of the *lsfA* homologue is incorrect for transcription of the promoterless *panB* gene. Nevertheless, fusion PI125 repeatedly colonized seedlings from a limited inoculum and β-galactosidase assays showed that *rhi-12* was transcriptionally active in the rhizosphere, albeit only 10-fold (Fig. 6). One possibility is that the 330 nucleotide region between the start of *lsfA* [the first gene of a sulphate-starvation-inducible operon (*ssu*; P. Vermeij, C. Wietek, A. Kahnert, T. Wuest and M. A. Kertesz, unpublished)] and promoterless *panB* contains a divergent promoter. A cosmid clone spanning this region was therefore obtained (G. M. Preston, unpublished), and the gene downstream of the 330 nucleotide intergenic region was found to encode a polypeptide with predicted similarity to OprE (an anaerobically induced porin protein from *P. aeruginosa;* Yamano *et al*., 1993). Unfortunately, the direction of transcription of *oprE* does not support the divergent promoter hypothesis, unless it results in transcription of an antisense RNA to the 3′ end of *oprE*. At present, there is no proof of this, however locus *np14*, which was identified during an IVET screen of *P. aeruginosa* genes induced during infection of neutropenic mice, is reported to encode an antisense RNA for OprE (Wang *et al*., 1996).

Trace elements, many of which are toxic to microbial life at micromolar concentrations, are important chemical constituents of the rhizosphere. *rhi-3* spans the junction between two ORFs that are similar to the 3′ end of *copR* and the 5′ end of *copS* from *P. syringae*. *copR* and *copS* are the regulator and sensor, respectively, of a copper-inducible operon that confers resistance to copper (Mills *et al*., 1993). As in *P. syringae*, *copR* and *copS* are translationally coupled, and a −1 frameshift is required for correct translation of *copS*. Preliminary experiments with PI011 show that *rhi-3* is also induced in response to trace amounts of copper (10 μM $CuSO_4$).

Loci *rhi-8* and *rhi-19* are similar to ORFs that encode proteins involved in drug and/or cation efflux and may play a role in protecting bacteria from harmful metabolites produced either by the plant or by other microorganisms. *rhi-8* shares 51% amino acid identity with *ragC* from *Bradyrhizobium japonicum,* which is a member of the AcrB/AcrD/AcrF family of efflux pumps. In *B. japonicum*, *ragC* is the fourth gene in an unusual operon that includes

a two-component sensing system and a sigma-32-like transcription factor (Narberhaus *et al.*, 1997). No similarity to other members of this operon was found in SBW25 when the DNA sequence from the opposite end of the fusion insert was compared with this region (N. Bertrand, unpublished.

A striking discovery was *rhi-18*, which shares 52% identity with HrcC from *Erwinia amylovora*, a component of the type III secretion pathway, or hypersensitive response and pathogenicity locus (*hrp*). HrcC proteins form channels within the outer membrane through which proteins secreted by the type III pathway pass and is absolutely required for type III secretion and for type III secretion-dependent phenotypes (Hueck, 1998). The DNA sequence from the opposite end of the 3 kb partial *Sau*3AI insert in PI143 revealed the presence of a gene that shares 63% identity with another *hrp*-encoded protein, HrcJ from *P. syringae* pv. *syringae*. HrcJ is also part of the secretion apparatus and is located in the inner membrane, where it is thought to function as a bridge across the periplasmic space connecting the protein secretion channels in the inner and outer membranes. The separation of *hrcC* and *hrcJ* by ≈ 3 kb is consistent with the arrangement of these genes in the group I *hrp* clusters of plant pathogenic bacteria (Alfano and Collmer, 1997).

Discussion

The data presented here demonstrate the utility of using an IVET strategy for studying adaptation of *P. fluorescens* to the rhizosphere environment. The pantothenate-based strategy was developed from first principles, and can be readily applied to the study of other plant-associated bacteria and environmental isolates that are amenable to basic genetic manipulation. IVET is essentially a promoter-trapping strategy, but its power and potential rests with its ability to trap promoters that are functionally active in the wild. This is because the selection system directly reports the activity of promoters (in their native chromosomal context) through their effect on growth. The secondary reporter has no role in '*in vivo*' selection, thus complications of artefactual expression that can arise through sole reliance on markers such as *lacZ* or *uidA* are avoided. Nevertheless, the secondary reporter is not inconsequential; it provides a means of eliminating constitutive promoters, it provides support for the environment-specific activity of fusions and it can be used for studies on spatial and temporal patterns of gene expression.

Twenty transcriptional units of *P. fluorescens* were identified that were induced in the rhizosphere of sugar beet seedlings. The 20 *rhi* genes were obtained by screening a library of 5000 clones, which represents ≈ 10% of the number of clones estimated to comprehensively survey the *P. fluorescens* genome for *rhi* genes. Even this modest collection of genes begins to provide an indication of the rhizosphere environment as perceived by *P. fluorescens*. The number of fusions displaying similarity to genes involved in nutrient acquisition, efflux and secretion, and stress response suggests a highly dynamic environment in which *P. fluorescens* is sending and receiving signals, competing for limiting nutrients and exposed to high levels of active oxygen species. This view of the rhizosphere augments the historical perspective, but genes such as *rhi-18* suggest the possibility of a highly specific and specialized interaction between plant root and rhizosphere colonist. Such an interaction, if mediated through a type III secretion pathway, might provide bacteria with access to a protected nutrient pool (He, 1998), and may even generate a signal sufficient to elicit induced systemic resistance in the plant. This possibility remains to be proven, but, if true, then exciting opportunities will open for the study and exploitation of rhizosphere-colonizing bacteria.

Efficient nutrient scavenging systems, combined with an ability to utilize a diverse array of nutrient sources, are likely to be crucial for competitive ability in the rhizosphere (Bakker *et al.*, 1993; Bolton *et al.*, 1993; Oresnik *et al.*, 1998). Studies of exudate collected from roots show the presence of various sugars, purines, nucleosides, organic acids, amino acids and vitamins (including pantothenate) (Rovira, 1969). However, when the availability of nutrients is 'seen through the eye' of a rhizosphere colonist, it is clear that nutrients, although present in exudate, may not be readily available to the microbial community (Streit *et al.*, 1996; Simons *et al.*, 1997). Pantothenate is undoubtedly limiting in the rhizosphere of sugar beet seedlings, and an initial screen of an extensive range of different auxotrophs for their ability to colonize the rhizosphere of both sugar beet and tomato seedlings (in both vermiculite and soil) showed that, with few exceptions, most amino acids are at limiting concentrations (P. B. Rainey, unpublished). It is therefore not surprising to find that one-quarter of the *rhi* genes have predicted similarity to genes involved in nutrient acquisition. Identification of genes with likely roles in the metabolism of organic acids (*rhi-2*) and xylose (*rhi-17*) indicate that these carbon sources are probably utilized by rhizosphere bacteria. Similarly, *rhi* genes with putative roles in uptake and transport of amino acids suggests that these compounds are scavenged by *P. fluorescens*, presumably to be used as sources of carbon and/or nitrogen. The presence of genes such as *rhi-4* (predicted to encode morphinone reductase) for which the natural substrate is unknown suggests that there is still much to learn about the nutritional status of the rhizosphere.

Elevated expression of genes encoding polypeptides with similarity to glutathione peroxidase and glyoxylase II indicates that rhizosphere-colonizing bacteria experience oxidative stress. This may arise from oxidative metabolism, from nutrient stress, or through exposure to

the superoxide and hydroxyl radicals produced by other members of the rhizosphere community. Efficient elimination of the harmful effects of oxidative stress is important for persistence in any environment, and it has recently been suggested that the cellular concentration of glutathione is an important factor in rhizosphere competence (Hultberg, 1998).

Many plant-associated microbes show resistance to heavy metals, especially copper that is essential in small amounts but is toxic in excess. The widespread occurrence of copper resistance mechanisms is often attributed to the use of copper sprays to control phytopathogenic pseudomonads, but they may also be widespread because of their role in maintenance of cellular copper homeostasis. Copper homeostasis is likely to be important in the rhizosphere given that the availability of trace metals is determined largely by pH, a factor that varies on a microscale along a root (Marschner, 1995). Interestingly, selection for *Salmonella* genes induced during infection revealed several genes with a role in metal transport and homeostasis, including one (*iviX*) that is Cu^{2+} inducible.

Of all the *rhi* genes, *rhi-18*, which encodes polypeptides with similarity to genes from the type III secretion pathway, is the most exciting. *rhi-18* is also the only *rhi* locus that has similarity to genes with a known role in colonization, albeit not of the rhizosphere but of intercellular spaces of plant leaves (Lindgren *et al.*, 1986; He, 1998). Sequence analysis of $\approx$ 25 kb of DNA flanking the fusion in PI143 has shown that SBW25 has an entire group I *hrp* cluster that bears overall similarity to the *hrp* cluster found in *P. syringae* pv. *syringae* (G. M. Preston and P. B. Rainey, unpublished). The type III secretion pathway has been described from a number of animal and plant pathogens, and more recently from the nitrogen-fixing symbiont *Rhizobium* (Freiberg *et al.*, 1997). This specialized pathway is induced in response to contact with eukaryotic host cells and is involved in the secretion of proteins into eukaryotic cells (Alfano and Collmer, 1997; Hueck, 1998). Although originally isolated as a pathogenicity determinant responsible for the delivery of virulence proteins into host cells, its involvement in the formation of nodules on tropical legumes by *Rhizobium* sp. NGR234 (Viprey *et al.*, 1998) suggests it may have a more general role in parasitism (Preston *et al.*, 1998). *P. fluorescens* SBW25 is not known to cause disease, in fact inability to cause disease on crop plants was a prerequisite for field release of a genetically modified derivative of SBW25 in 1994 (Bailey *et al.*, 1995; Thompson *et al.*, 1995). Work in this laboratory has confirmed its non-pathogenic phenotype and has shown SBW25 to be incapable of eliciting the hypersensitive response (HR) on sugar beet, tomato, *Arabidopsis* or tobacco. Additional support for its non-pathogenic phenotype comes from the fact that SBW25 is able to protect seedlings against disease caused by fungal pathogens. The existence, therefore, of a type III pathway in non-pathogenic *P. fluorescens* raises important questions concerning the nature of the association that this and similar bacteria form with both plant roots and/or other members of the rhizosphere community. It also serves to add to the growing number of studies that indicate that the boundaries between pathogen and saprophyte are not as distinct as once believed (Preston *et al.*, 1998). We are currently attempting to determine the function of the pathway through mutagenesis and expression studies.

Application of IVET strategies to the study of pathogenicity in mammalian hosts has consistently resulted in the discovery of ORFs of unknown function, some of which lack any statistical similarity with sequences in DNA and protein databases. Given that each sequenced genome of a microorganism possesses a substantial number of genes that bear no similarity to previously identified genes, this result is not surprising (Strauss and Falkow, 1997). Techniques such as IVET provide evidence that these novel ORFs are transcriptionally active in the rhizosphere and provide impetus for further study. Of the six fusions to which no putative function could be assigned, only one showed any similarity to an ORF from *P. aeruginosa* — a bacterium that is closely related to *P. fluorescens*. This suggests the possibility that these genes may be environment specific with a unique role in rhizosphere colonization.

With the exception of *rhi-3* (Cu^{2+} inducible), the signals activating transcription of *rhi* genes remain to be determined. By selecting *rhi* fusion strains on minimal medium (as opposed to a complete medium), many of the housekeeping genes specific to low nutrient environments, such as amino acid metabolism genes, ought to be avoided. It is therefore notable that *rhi* genes with predicted roles in nutrient acquisition, particularly the putative amino acid transporters, were not active on either minimal medium (no amino acids) or LB (replete in amino acids). Their lack of response to typical signals suggests that these *rhi* genes have a specialized contribution to cellular metabolism that lies beyond the ordinary housekeeping amino acid metabolism machinery. Consistent with this is the observation that *rhi-10* shows greatest similarity to an uncharacterized region of the *P. aeruginosa* genome that is distinct from the known (*BraCDEFG*) high-affinity amino acid transport pathway. Interestingly, *rhi-14* (predicted histidine transporter) showed a low level of induction when inoculated into the rhizosphere (Fig. 7), which may reflect the fact that it requires a highly specific signal for activation — possibly intimate contact with the plant root or another member of the rhizosphere community. The same is possibly true of *rhi-12* and *rhi-19*, both of which also showed low levels of rhizosphere induction. The potential for such a signal to activate transcription

may have been denied by the fact that in the rhizosphere assay the fusion strains were inoculated onto 2-week-old seedlings rather than being allowed to establish from the seed.

A major role for recombination in the evolution of bacteria is indicated by genome sequence data from a range of bacteria (Groisman and Ochman, 1996; Conner *et al.*, 1998; Lawrence and Ochman, 1998). The fact that seven *rhi* genes are more similar to genes from non-*Pseudomonas* species suggests that horizontal gene transfer may also be an important force in the evolution of the SBW25 genome. A particularly striking example comes from strain PI009, in which the gene adjacent to *rhi-2* is 45% identical (over 131 amino acids) to a hypothetical protein from *Mycobacterium tuberculosis* (Rv2024c); there are no other sequences in the databases with any significant similarity to this region. Interestingly, in several instances, the species to which a given *rhi* gene shows greatest similarity is another member of the rhizosphere community. For instance, *rhi-2*, *rhi-18*, *rhi-8* and *rhi-1* have no homologue in *P. aeruginosa*, but share similarity to genes found in *Rhodobacter capsulatus*, *Erwinia amylovora*, *Bradyrhizobium japonicum* and *Streptomyces peucetius* respectively. This suggests the possibility of 'niche-specific traits', i.e. traits shared by bacteria that occupy the same niche irrespective of their phylogenetic position.

Unravelling the phenotypic and genotypic causes of ecological performance in any organism is a complex and open-ended problem (Lenski, 1995). The genes-up approach advocated here seeks to identify genes likely to enhance ecological performance on the basis of their elevated level of expression in the rhizosphere. Current work is directed toward completing analysis of the *rhi* gene status of the SBW25 genome, determining the function and ecological role of selected *rhi* gene products and the contribution of each to ecological performance. Once this is complete and supplemented with knowledge of the effect of specific rhizosphere signals on the expression of fitness-enhancing traits, then predicting the performance of a PGPR in a given rhizosphere environment may be possible.

Experimental procedures

Bacterial strains, plasmids and media

Bacterial strains and plasmids used in this study are described in Table 2. The ancestral strain of *P. fluorescens* SBW25 was isolated from field-grown sugar beet at the university farm, Wytham, Oxford, in 1989 (Thompson *et al.*, 1995). *P. fluorescens* and *E. coli* were routinely grown in LB broth (or agar) with antibiotics as needed, at 28°C and 37°C respectively. *P. fluorescens* was also cultured on King's medium B (KB; King *et al.*, 1954) or minimal (M9) medium (Sambrook *et al.*, 1989). Antibiotics were used at the following concentrations: ampicillin, 100 μg ml^{-1} for *E. coli*; pipercillin, 125 μg ml^{-1} for *P. fluorescens*; tetracycline, 25 μg ml^{-1} for *P. fluorescens* in LB and 12.5 μg ml^{-1} in M9 plates and LB broth; kanamycin, 75 μg ml^{-1} for *E. coli* and *P. fluorescens*; streptomycin, 100 μg ml^{-1} for *P. fluorescens*. Antibiotics were obtained from Sigma. CFC supplement (Difco) was used to select for *P. fluorescens* recovered from the rhizosphere and was used at full strength in KB and LB and half strength in M9. Pantothenate [pantothenic acid (hemicalcium salt)] was added at 0.1 mM when required.

Germination, inoculation and cultivation of sugar beet seedlings

Coated sugar beet seeds (*Beta vulgaris* var. Amethyst) were germinated and cultivated in 5 ml scintillation vials containing non-sterile vermiculite as a growth substrate. Seeds were covered with vermiculite and germinated (one seed per vial) in a plant growth cabinet at 20°C with a 16 h light/8 h dark cycle (within 24 h of seed sowing, the average bacterial count on uninoculated seeds was $\approx 10^8$ cells) For inoculation with bacteria, cultures were grown overnight and were washed twice in distilled water. The optical density (A_{600}) was adjusted to 0.05 and a 200 μl sample was added to 5 ml of water. Seeds were placed in this dilute suspension for 5 min, they were then removed and excess moisture blotted with tissue before placing single seeds in vermiculite-containing vials. Seeds treated in this way received $\approx 10^4$ bacterial cells. To harvest cells from the rhizosphere, the photosynthetic parts of the seedling were removed, and the entire contents of each vial (the rhizosphere) added to 25 ml Sterilin tubes containing $\approx$ 40 glass beads and 5 ml one-quarter strength Ringers solution. The rhizosphere (roots plus attached vermiculite) was vortexed for 1 min at high speed and viable cell counts were determined using the drop method (Miles and Misra, 1938).

DNA manipulations and sequencing

General recombinant DNA techniques were performed using standard methods (Sambrook *et al.*, 1989). Restriction and modifying enzymes were obtained from BRL and NEB. Southern hybridization was performed using ECL (Amersham). DNA was sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) with (α-^{35}S)-dATP (Amersham), or using Big-Dye terminators (ABI) on an automated sequencer model ABI310 (Perkin Elmer). Details of oligonucleotides for vector construction are available upon request.

Transposon mutagenesis

PF126 was obtained from a screen of auxotrophic mutants of *P. fluorescens* SBW25 (to be reported fully elsewhere). Mutants were generated by conjugation using the suicide vector pUT mini-Tn*5 xylE*. Donor (S17-1 λ*pir*) and recipient (heated to 45°C for 15 min immediately before the conjugation) were mixed 1:4 and incubated for 6 h on LB plates before selecting transconjugants on KB supplemented with kanamycin and ampicillin. Auxotrophs were screened by replica plating

Table 2. Bacterial strains and plasmids used in this work.

Strain plasmid	Revelant properties	Source/reference
P. fluorescens strain		
SBW25	Wild type	Rainey and Bailey (1996)
SBW25-Sm	Spontaneous SM′ derivative of SBW25	This work
PF126	Pantothenate auxotroph of SBW25 (*panB*::mini-Tn*5 xylE*)	This work
SBW25Δ*panB*	Pantothenate auxotroph of SBW25 (*panB* gene deleted)	This work
SBW25Δ*panB*-Sm	Spontaneous SM′ derivative of SBW25Δ*panB*	This work
E. coli strain		
XL1 MR	Δ(*mrcA*) 183Δ(*mcrCB-hsdSMR-mrr*) 173 *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*	Stratagene
XL1 MRF′	Δ(*mrcA*) 183 Δ (*mcrCB-hsdSMR-mrr*) 173 *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proABlacI′ ZDM 15* Tn *10* (Tc′)]	Stratagene
DH5α*λpir*	*supE44* Δ*lacU169* (ϕ80*lacZDM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*, *λpir*	M. Mahan
S17-1 *λpir*	Tp′ Sm′ *recA, thi, pro, hsdR*$^{-}$*M*$^{+}$, RP4-2: Mu-Km::Tn7, *λpir*	Simon *et al.* (1983)
Hfr3000 YA139	*panB* mutant derivative of K12	Cronan (1980)
Plasmid		
pBluescript (SK^{+})	Ap′ cloning vector	Stratagene
pBR322	Ap′, Tc′ cloning vector; source of Tc′ gene	Bolivar *et al.* (1977)
PIJ3200	Broad host range cosmid, Tc′	Liu *et al.* (1990)
pUT mini-Tn5 *xylE*	Delivery plasmid for mini-Tn*5 xylE*	Herrero *et al.* (1990)
pJMSA2	Source of *′lacZY* for pIVETP	J. Sanchez Romero
pPS126	piJ3200 cosmid clone (~25 kb) containing pantothenate biosynthetic locus (See Fig. 3)	This work
pPS126-18	pIJ3200 containing 1.8 kb *Pst*I fragment from pPS126 (see Fig. 3)	This work
pSK18	pBluescript containing 1.8 kb *Pst*I fragment from pPS126 (see Fig. 3)	This work
pIVET2	*thyA*-based IVET vector; Ap′, *′lacZY*	Mahan *et al.* (1993)
pIVET-L	pIVET2 without *thyA* gene and with modified MCS	This work
pUIC3	Universal IVET contruct (see Fig. 4)	This work
PIVETP	*panB*-based IVET vector (see Fig. 4)	This work
pSK-Sm4	4 kb *Sma*I fragment from pPS126 (see Fig. 3)	This work
pSK-SP23	2.3 kb *Sma*I/*Pst*I fragment from pSK-SM4 cloned in pBluescript	This work
PSK007	*panB* deletion of pSK-18	This work
pSK-Sa85	8.5 kb *Sal*I fragment from pPS126 (see Fig. 3) cloned into the *Sma*I site of pBluescript	This work
pSK009	pSK-SP23 with the *panB* deletion from pSK007 cloned into the unique *Pst*I site. Insert excisable as a *Sma*I fragment	This work
pSK010	PSK-SP23 with the internal 4 kb *Sma*I fragment replaced by the *Sma*I fragment from pSK009	This work
pDPB011	pIVET-L containing the *Spe*I/*Sal*I insert from pSK010 cloned into *Spe*I/*Sal*I-digested pIVET-L	This work

on M9 medium, and precise deficiencies were determined by auxanography (Davis *et al.*, 1980). Fitness of PF126 in the sugar beet rhizosphere was determined by direct competition with a streptomycin-resistant derivative of the ancestral genotype. Mutant and ancestor were inoculated onto seeds at a ratio of 1:1 and all assays were performed in triplicate. Cells were harvested from seedlings after 7, 14 and 21 days and were plated on appropriate selective media. PF126 was selected on KB supplemented with pantothenate, CFC and kanamycin. Its identity on the basis of the *xylE* gene was confirmed by spraying plates with 1% catechol. SBW25-Sm was selected on M9/glucose supplemented with streptomycin and CFC.

Cosmid library construction

A cosmid library of *P. fluorescens* SBW25 was constructed in cosmid pIJ3200 following standard procedures. Insert DNA was prepared by partial *Sau*3AI digestion of SBW25 genomic DNA, which was size fractionated on an agarose gel. Fragments for 23–28 kb were extracted using Qaiex beads and ligated with *Bam*HI-digested pIJ3200. The ligated DNA was packaged into λ phage heads using Gigapack III Gold packaging extract (Stratagene) according to the manufacturer's instructions. The phage particles were transduced to *E. coli* XL1 MR, and the cosmid-containing cells were selected on LB agar plates containing tetracycline.

Complementation of PF126, cloning and sequencing of the pantothenate locus

The cosmid library was mobilized *en masse* to PF126 by conjugation using helper pRK2013 and a cosmid containing the pantothenate biosynthetic locus, which was obtained by selecting for PF126 colonies able to grow on M9 agar. A single cosmid, pPS126, was isolated, which restored PF126 to prototrophy. To locate the complementing gene(s), pPS126 was shotgun cloned in pIJ3200 after digestion with *Pst*I. The ligation mixture was transformed into PF126, where subclones able to complement the pantothenate lesion were selected

by complementation. The minimal complementing unit was a 1.8 kb *Pst*I fragment, which was located centrally within pPS126 (see Fig. 3). Careful investigation of the PF126 (pPS126-18) colonies showed that only a fraction (< 1%) were prototrophic. Subsequent work showed that PF126 carried mini-Tn*5* within *panB* and that the polar effects of the transposon prevented complementation by pPS126-18 in *trans*. However, complementation did occur in *cis* as a result of homologous recombination between pPS126-18 and the chromosomal *panB* locus, which resulted in integration of pPS126-18 and restoration of the wild-type operon. The 1.8 kb *Pst*I fragment was cloned into pBluescript (pSK-18), and nested deletions were generated from both ends of the insert. Both strands were sequenced.

*Construction of SBW25*Δpan B

Plasmid pSK-18 was the starting point for the construction of the *panB* deletion. The deletion was generated by SOE-PCR (Horton *et al*., 1989) and was accomplished in two stages. First, two PCR reactions were performed; one to amplify the ≈ 830 nucleotides upstream of the *panB* initiation codon and the second to amplify the ≈ 250 nucleotides of *panC* downstream of (and including) the initiation codon. The antisense primer for the first reaction included 19 nucleotides, which were complementary to the start of the *panC* ORF. To complete construction of the deletion, the two amplified regions were SOE-en together by a third PCR reaction, which used the sense and antisense primers from the first and second reaction respectively. The resulting fragment was cloned into the *Pst*I site of pBluescript. This plasmid was designated pSK007 and was sequenced to check for errors (see Fig. 3 for precise details of the fusion).

In order to provide sufficient DNA either side of the deletion for subsequent homologous recombination, several cloning steps were necessary. First, the 4 kb *Sma*I fragment and the 8.5 kb *Sal*I fragments from pPS126 were cloned into *Sma*I-digested pBluescript to generate pSK-Sm4 and pSK-Sa85 respectively. pSK-Sm4 was further subcloned to generate pSK-SmPs23: pSK-Sm4 was digested with *Sma*I and *Pst*I and the 2.3 kb fragment cloned into *Sma*I/*Pst*I-digested pBluescript. The insert from pSK007 was then excised with *Pst*I and inserted into the *Pst*I site of pSK-SmPs23 to generate pSK009. Finally, pSK010 was generated, which consisted of pSK-Sa85 in which the internal 4 kb *Sma*I fragment was replaced with the 3 kb (Δ*panB*) *Sma*I fragment from pSK009.

To introduce the *panB* deletion into the SBW25 chromosome, a two-step allelic replacement strategy was used. For this purpose, pDPB011 was generated, consisting of the 7.5 kb fragment from pSK010, excised as a *Spe*I/*Sal*I fragment and cloned into pIVET-L. pDPB011, which cannot replicate in *Pseudomonas*, was mobilized by conjugation into SBW25, where integration by a single homologous recombination event was selected by plating on LB agar containing pipercillin and Xgal. Blue-coloured, pipercillin-resistant colonies containing both intact and deleted versions of *panB* (confirmed by Southern hybridization) were grown without selection in LB broth to select for the second homologous recombination event. After two successive 24 h subcultures, cells were plated on LB agar containing Xgal, and white colonies were screened for loss of pipercillin resistance. Approximately 2% of the colonies were white and sensitive to pipercillin and these were replica plated to M9 agar to check for pantothenate auxotrophy. Approximately 40% of these colonies were auxotrophic for pantothenate. Loss of *panB* and vector sequence was confirmed by Southern hybridization. To check that the deletion had not affected the function of the remaining genes of the pantothenate operon, pPS126-18 (containing *panB*) was introduced into SBW25Δ*panB*, where it was able to restore the deletion mutant to prototrophy (when present in *trans*). Fitness of SBW25Δ*panB* in the rhizosphere was determined by a competitive assay using a spontaneous streptomycin-resistant derivative.

Construction of pIVETP

Initially, I planned to construct pIVETP by replacing the *thyA* gene of pIVET2 (Mahan *et al*., 1993) with promoterless *panB*, however the promoterless *lacZY* operon in pIVET2 was active in *P. fluorescens* — even in the absence of an active promoter. To overcome this problem and to tailor the vector for *Pseudomonas*, the IVET vector was reconstructed from pGP704 (Fig. 4). Promoterless *lacZY* was excised from pJM501 and was cloned into *Sal*I/*Sph*I-digested pGP704. The tetracycline resistance gene from pBR322 was inserted into the unique *Sph*I site, and a multiple cloning site, which included translational stop codons in the three forward reading frames, was incorporated. To complete construction of pIVETP, promoterless *panB* was obtained by PCR from pSK-18 and inserted into the unique *Spe*I site (Fig. 4).

Construction of P. fluorescens *genomic libraries in pIVETP and integration into SBW25*Δpan B

Genomic libraries were constructed with DNA obtained from SBW25. Genomic DNA was partially digested with *Sau*3AI and was size fractionated by agarose gel electrophoresis. Fragments 3–5 kb in length were extracted (Qaiex), ligated to *Bgl*II-digested alkaline phosphatase-treated pIVETP and transformed into *E. coli* S17-1 λ*pir*. Transformants were selected on LB agar containing tetracycline and grouped in pools of ≈ 250 colonies. IVET fusion plasmids from individual pools were transferred to SBW25Δ*panB* by *en masse* conjugation, and transconjugants containing integrated fusions were selected on M9 agar containing tetracycline, pantothenate and Xgal.

Screening of P. fluorescens *libraries for* rhi *genes*

Five pools of fusion strains, representing ≈ 5000 clones, were screened for rhizosphere-induced genes (*rhi* genes) by inoculating 50 seeds with 10^3 bacteria (10 seeds per pool). After 14 days of selection, bacteria were recovered from the rhizosphere and colonies containing fusions to putative *rhi* genes (Lac^- colonies) were selected on M9 agar containing tetracycline, pantothenate and Xgal. To maximize the efficiency of library screening, the initial inoculum added to seeds was maintained at a low level. This ensured maximum opportunity for selection, but also ensured that each seed received on average one *rhi* fusion strain. When *rhi* fusion strains were recovered, only two colonies were selected for further

analysis because of the high probability that *rhi* fusions from the same rhizosphere were siblings.

White colonies were checked for auxotrophy on M9 and plasmids were recovered by conjugative cloning (Rainey *et al*., 1997). At its simples, this involved spreading 1:1:1 mixtures of overnight cultures of SBW25Δ*panB* fusion strain, *E. coli* (pRK2013) helper and *E. coli* DH5α λ*pir* on LB agar plates containing tetracycline, which were incubated at 39°C to select against *P. fluorescens*. Plasmids were purified from DH5α λ*pir* and were double digested with *Eco*RI and *Bam*HI to check for siblings. Fusions were sequenced directly using a primer to the 5′ end of the promoterless *panB* gene (5′-GTCAGGGTAATGTCTGGCATG). In some instances, sequence was obtained from the opposite end of the fusion using a primer to the 3′ end of the *bla* gene (5′-CAGGGTTATTGTCTCATGAGCG). In each instance, more than 600 bp of sequence was obtained.

Assay for β-galactosidase activity

β-galactosidase was assayed essentially as described by Miller (1972), except 4-methylumbelliferyl-β-D-galactoside was used as the substrate and the product, 7-hydroxy-4-methylcoumarin (4 MU), was detected using a fluorometer (reaction monitored at 460 nm with 365 nm excitation wavelength). To determine the β-galactosidase activity of SBW25Δ*panB rhi* fusion strains in the sugar beet rhizosphere, individual fusion strains were inoculated into the rhizosphere of 2-week-old seedlings. Fusion strains were recovered after 24 h by suspending the entire rhizosphere (contents of each vial) in a 25 ml Sterilin tube containing 3 ml water. Each rhizosphere was shaken gently for 5 min (without glass beads) and then left to stand for 1 min before removing 1 ml of bacterial suspension. This was concentrated 10-fold by centrifugation and the number of bacteria was determined by plating. Results were expressed as aM 4 MU cell^{-1} min^{-1}. Uninoculated controls showed no significant background of β-galactosidase activity.

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