

Use of differential fluorescence induction and optical trapping to isolate environmentally induced genes

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Summary

The techniques of differential fluorescence induction (DFI) and optical trapping (OT) have been combined to allow the identification of environmentally induced genes in single bacterial cells. Designated DFI-OT, this technique allows the *in situ* isolation of genes driving the expression of green fluorescent protein (Gfp) using temporal and spatial criteria. A series of plasmid-based promoter probe vectors (pOT) was developed for the construction of random genomic libraries that are linked to *gfpUV* or *egfp*. Bacteria that do not express Gfp on laboratory medium (i.e. non-fluorescent) were inoculated into the environment, and induced genes were detected with a combined fluorescence/optical trapping microscope. Using this selection strategy, rhizosphere-induced genes with homology to thiamine pyrophosphorylase (*thiE*) and cyclic glucan synthase (*ndvB*) were isolated. Other genes were expressed late in the stationary phase or as a consequence of surface-dependent growth, including *fixND* and *metX*, and a putative ABC transporter of putrescine. This strategy provides a unique ability to combine spatial, temporal and physical information to identify environmental regulation of bacterial gene expression.

Introduction

The *in situ* physiology of soil microbes is difficult to analyse because the complex natural environment cannot

be replicated in the laboratory. To overcome this difficulty, several new techniques have been developed to measure microbiological activities *in situ* (Osbourn *et al.*, 1987; Mahan *et al.*, 1993; Hensel *et al.*, 1995; Valdivia and Falkow, 1996; 1997; Pelz *et al.*, 1999; Rainey, 1999; Ramos *et al.*, 2000). These include signature-tagged mutagenesis (STM), *in vivo* expression technology (IVET) and differential fluorescence induction (DFI) (for reviews, see Chiang *et al.*, 1999; Handfield and Levesque, 1999; Lee and Camilli, 2000). Each has its particular benefits and weaknesses and has a role in investigating gene induction in the environment.

In DFI, expression of a modified green fluorescent protein (Gfp) reporter fusion is linked to a fluorescence-activated cell sorter (FACS) (Valdivia and Falkow, 1996; 1997). The benefits of DFI include the automated screening of large populations and the ability to alter the fluorescence window to detect changes in expression levels rather than simply identifying tightly regulated promoters. However, disadvantages of DFI include problems sorting bacteria, which are at the low end of the detection capabilities of flow cytometers (Valdivia and Falkow, 1996), and the difficulty of isolating bacteria from naturally fluorescent environments.

Gfp has a number of advantages over other reporters of gene expression. It is detectable in single cells using a microscope and requires no cofactors (Gage *et al.*, 1996; Cirillo *et al.*, 1998). We considered that a powerful strategy would be to produce a versatile promoter probe vector linked to *gfp* for use on agar or microtitre plates or under the microscope and that this precision should be combined with IVET/DFI-like strategies to enable spatial information from microscopy to be linked with the ability to select genes specifically induced in the environment. The ability to isolate single cells from a population has been made possible by the recent development of optical trapping microscopes (Ashkin, 1997; Allaway *et al.*, 2000). Optical trapping uses the property of coherent light to produce radiational pressure, sufficient to hold a bacterium within the light beam (Ashkin and Dziedzic, 1987; 1989; Ashkin *et al.*, 1987).

Combining a DFI strategy with optical trapping (OT) has allowed the development of a system that enables the identification and isolation of single cells expressing environmentally induced gene fusions under a microscope for further characterization. Here, we describe the construction and testing of this strategy, designated

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DFI-OT, with respect to identifying environmentally induced promoters in the agriculturally important rhizosphere bacterium, *Rhizobium leguminosarum*.

Results and discussion

Development of a promoter probe vector for optical trapping

Our aims in this project were twofold. First, we wanted to construct a promoter probe vector that allows the easy production of large genomic libraries in a broad-host-range plasmid linked to Gfp. Although many promoter probe vectors exist, some of which are linked to Gfp, these are usually unsuitable for the production of large random genomic libraries. Such a vector must have no background expression of the reporter gene, and it must facilitate the production of libraries with tens of thousands of independent clones. Secondly, we wanted to use such libraries both on their own under demanding selection criteria and in conjunction with an optical trap (DFI-OT).

The small, broad-host-range vector pBBR1-MCS-5 (Kovach *et al.*, 1995) was chosen because it has a medium copy number, is mobilizable in the presence of RK2 *tra* genes when present *in trans*, is stably maintained in Gram-negative bacteria and the entire nucleotide sequence is known. It also encodes gentamicin resistance making it suitable for studies in a wide range of bacteria. The reporter gene *gfpuv* was chosen because it has an 18-fold increase in fluorescence relative to the wild-type *gfp* but retains the latter's excitation and emission maxima of 395 and 510 nm respectively (Crameri *et al.*, 1995). The large difference in excitation and emission maxima makes it easy to visualize fluorescence on agar plates using simple long-wavelength transilluminators. To make *gfpuv* usable for a library construction, its *SalI* site was removed by site-directed mutagenesis. A *SalI* site was incorporated into the polylinker of pOT1 to allow cloning of *Sau3AI*-digested genomic DNA after both vector and insert are partially infilled (see *Experimental procedures*). This strategy minimizes the proportion of both multimeric insertions and self-ligated vector present in the final library. Translational stop sites were inserted in each of the forward reading frames downstream of the *SalI* cloning site to prevent the formation of translational fusion proteins between insert DNA and Gfp-UV. Rare cutting restriction enzyme sites, *PmeI* and *PacI*, were introduced to flank the *SalI* cloning site, enabling cloning into the pUC derivative pNEB193 (New England Biolabs). Transcriptional termination sequences were cloned 5' and 3' to the multiple cloning site and *gfpUV* respectively (final vector EMBL AJ131020).

To extend the usefulness of the original pOT1 vector for FAC sorting and DFI analysis, a derivative was made

(pOT1e) by exchanging *egfp* for *gfpuv*. eGfp-1 has chromophore mutations at F64L and S65T, which red shifts the excitation maximum to 488 nm and increases the fluorescence 35-fold relative to wild-type Gfp (Cormack *et al.*, 1996). A second derivative of the basic pOT1 vector, designated pOT2 (EMBL AJ310442), has been made for the introduction of other reporter genes in tandem with Gfp, enabling IVET-type strategies to be used rather than DFI-OT technologies. Oligo priming sites pOT forward and pOT reverse are useful for sequencing. It should be noted that the *SpeI* site is very close to the ribosome binding site of all *gfp* genes and should not be used for cloning. The key features of the pOT vectors are shown in Fig. 1. All the vectors were tested for correct induction of *gfp* by cloning a *lacZ*, *dctA* or xylose kinase promoter into the polylinker (data not shown).

Construction of a genomic library

Rhizobium leguminosarum strain 3841 genomic DNA was cloned into pOT1, and $\approx 72\,500$ separate colonies were recovered. Polymerase chain reaction (PCR) screening of 55 colonies showed that the distribution of insert sizes ranged from < 0.5 kb to 3.5 kb with an average of 1.6 kb. The library was conjugated into strain 3841, and colonies were selected on chemically defined minimal medium. To establish whether the *R. leguminosarum* genomic library works effectively, several thousand colony-forming units were screened for promoters induced by phosphate starvation or by growth on xylose as the sole carbon source. Numerous fluorescent colonies were obtained for each condition tested, and three plasmids induced by phosphate limitation (pRU582–pRU584) and one by growth on xylose (pRU604) were isolated and sequenced with pOT reverse primer (Table 1). The first phosphate-regulated gene is *phoC* (pRU582), based on 88% identity over the first 83 amino acids to *phoCDET* of *Sinorhizobium meliloti*, which codes for a high-affinity phosphate uptake system (Voegelé *et al.*, 1997). The second is *phoA* (pRU583), based on around 50% identity to a broad range of eukaryotic alkaline phosphatases, including 54% identity to residues 34–67 of the human protein. The third (pRU584) has 77% identity to a putative iron-binding protein identified in *S. meliloti* (accession number AF030523). These three plasmids were also conjugated into *S. meliloti* 1021 and a *phoB* mutant strain RmH406. All three fusions were induced by phosphate limitation in strain 1021, but none was induced in RmH406 (data not shown). These genes are therefore likely to be PhoB regulated in *R. leguminosarum*. Interestingly, the *phoCDET* operon would not have been detected as induced by phosphate starvation using a knock-out strategy, because mutations in *phoCDET* cause its constitutive expression (Bardin and Finan, 1998). The xylose-inducible fusion

(pRU604) has 76% identity over the first 17 residues of xylose kinase from *Klebsiella pneumoniae*.

Identification of surface- and stress-induced genes

The ease with which Gfp-UV can be mass screened on agar plates and in liquid culture in microtitre plates suggested that it should be possible to compare gene expression in liquid and in solid culture. The expression of

gfp was examined in 11 000 colonies replica plated onto agar and into liquid medium. Twenty-four isolates were isolated that consistently gave higher gene expression on plates. Cells were also assayed after 4, 7 and 10 days growth. Two strains, RU1188, containing pRU498, and RU1256, containing pRU530, showed a 5.2-fold and 3.8-fold increase in expression over the 4–10 day period respectively. This increased expression indicates that these may contain promoters active during extreme starvation. One translation of the DNA proximal to *gfpuv* in plasmid pRU498 has high identity to homoserine O-acetyltransferases, including a predicted homologue in *Bacillus cereus* (52% identity over 67 residues) and MetX in *Leptospira meyeri* (43% identity over 67 residues) (Table 1). MetX is the first enzyme in methionine biosynthesis in a number of organisms and has been found to be an important virulence determinant in many plant–pathogen interactions. More relevant here, perhaps, is the observation that methionine prototrophy is required for bacterial fitness in stressful environmental conditions and may have a role to play in osmotolerance (Andersen *et al.*, 1998). One translation of the insert DNA in plasmid pRU530 has 96% identity over 53 residues to *fixN* of *R. leguminosarum*, which constitutes a component of the high-affinity CBB3 cytochrome oxidase. The *fixN* gene is part of the *fixNOQP* operon found on pSym, and its expression occurs both in the symbiotic zone of the root nodule and under free-living microaerobic conditions (Schluter *et al.*, 1997).

Most plasmids from the 24 showing differential fluorescence on agar and in broth were not induced further by prolonged growth in broth. Nine strains showing strong induction were sequenced (Table 1). BLAST searches did not reveal any similar sequences in the GenBank database for the insert DNA in either pRU496 or pRU514. The insert sequence in plasmid pRU497 codes

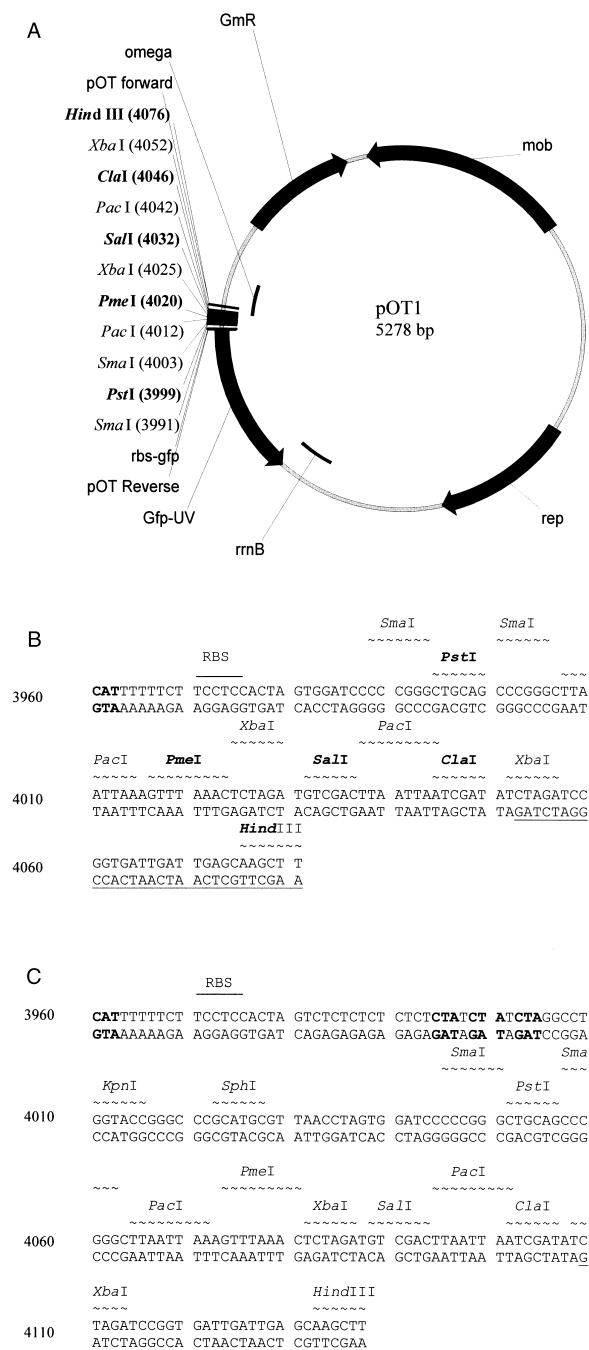


Fig. 1. Map of pOT1.

A. The details of construction of the cloning vector, pOT1, are in *Experimental procedures*. The final construct has *gfpuv* flanked by the omega and *rrnB* transcriptional terminators. An artificial ribosomal binding site (RBS) was synthesized in the 5' primer to *gfpuv*.

B. The polylinker of pOT1.

C. The polylinker of pOT2. Unique restriction enzymes are shown in bold; the other sites shown are duplicated to allow removal of cloned inserts but are only found in the polylinker. There is an additional unique *SpeI* site next to the RBS, but this has been omitted because cloning into this site has proved problematic for proper expression of *gfpuv*. The ATG start of *gfp* and the clustered stop codons in all three reading frames of pOT2 are shown in bold. Stop codons do occur in all three reading frames for pOT1 downstream of the *SalI* cloning site, but these have been omitted for clarity. The end of the omega transcriptional terminator is underlined. Although the *HindIII* site is in the terminator sequence, it is not part of the stem–loop, indicating that it can still be used as a cloning site. The full sequences of these vectors are available, pOT1 (EMBL AJ131020) and pOT2 (EMBL AJ310442). rep, replication; mob, mobilization.

Table 1. Environmentally induced genes.

Plasmid	Gene identity and selection condition
pRU496	No known homology (plate)
pRU497	Conserved ORF of unknown function (plate)
pRU498	52% over residues 3–68 of homoserine O-acetyltransferase (<i>metX</i>) of <i>B. cereus</i> (plate)
pRU500	40% over residues 182–230 of 16S rRNA dimethyltransferase of <i>Helicobacter pylori</i> (plate)
pRU504	39% over residues 8–97 of thiamine phosphate pyrophosphorylase (<i>thiE</i>) of <i>A. fulgidus</i> (plant rhizosphere)
pRU514	No known homology (plate)
pRU517	No known homology but is present 250 bp upstream of the methyl-accepting chemotaxis receptor (<i>mcpD</i>) of <i>R. leguminosarum</i> (plate)
pRU528	32% over residues 101–217 of a component of the putrescine transporter (<i>potH</i>) of <i>E. coli</i> (plate)
pRU529	34% over residues 391–459 to phytochrome of <i>Synechocystis</i> sp. (plate)
pRU530	96% over residues 1–53 of a component of the high-affinity terminal oxidase (<i>fixN</i>) of <i>R. leguminosarum</i> (plate)
pRU582	88% to the first 83 amino acids of alkaline phosphatase (<i>phoC</i>) from <i>S. meliloti</i> (phosphate limitation)
pRU583	54% to residues 34–67 of human alkaline phosphatase (phosphate limitation)
pRU584	77% to residues 25–105 of a putative iron-binding protein from <i>S. meliloti</i> (GenBank AF030523) (phosphate limitation)
pRU604	76% to the first 17 residues of xylose kinase of <i>Klebsiella pneumoniae</i> (xylose induction)
pRU659	75% over residues 1080–1233 of cyclic β -(1,2)-glucan synthase (<i>ndvB</i>) of <i>S. meliloti</i> (rhizosphere)

The selection conditions are indicated in brackets after each entry.

for a putative protein with similarity to a conserved open reading frame (ORF) of unknown function present in *Escherichia coli* (YEIH), *Haemophilus influenzae* and *Archaeoglobus fulgidus*. The insert in pRU500 has similarity (40% over 48 residues) to an rRNA dimethyltransferase from *Helicobacter pylori*. The insert in pRU517 does not code for a known protein, but the *gfpuv* fusion junction is 250 bp downstream from *mcpD* (codes for a methyl-accepting chemotaxis protein) in *R. leguminosarum* (Yost *et al.*, 1998) and may be part of an operon. The sequence in pRU528 potentially codes for a protein that has significant identity (32% over 117 residues) to the inner membrane protein PotH, which is a component of an ABC transporter of putrescine in *E. coli* (Pistocchi *et al.*, 1993). Finally, the insert sequence in plasmid pRU529 potentially codes for a protein similar to a phytochrome from *Synechocystis* sp. (34% identity over 111 residues). Genome sequencing has revealed a sequence for a phytochrome-like molecule in *Pseudomonas aeruginosa*. The possible existence of a phytochrome-like molecule in *R. leguminosarum* and *P. aeruginosa* is intriguing, although its function is unknown.

Isolation by DFI-OT of promoters induced in the pea rhizosphere

Our principal aim in developing the pOT vectors was to use them in combination with an optical trap in the technique of DFI-OT. To isolate environmentally regulated promoters by DFI-OT requires a library with no constitutive promoters. Therefore, 15 075 colonies were inoculated into 96-well microtitre plates and onto agar plates containing chemically defined minimal broth. Those that did not fluoresce on either agar or in broth were pooled to provide the *in vitro* silent library (11 841 colonies) to test in

the rhizosphere. A Nikon Optiphot epifluorescence microscope was modified for optical trapping using an infrared (IR) Nd YAG laser (1064 nm) (see *Experimental procedures*, Fig. 2). To test this system and identify pea plant-dependent gene expression, the *in vitro* silent library was inoculated onto pea seedlings grown in sterile vermiculite. After 7–10 days, the filtrate from the ground roots was observed with the optical trapping microscope under UV excitation. Of 11 fluorescent cells trapped and incubated in TY broth, three grew. To establish whether these cultures had grown from a single trapped cell or were heavily contaminated, PCR screening was performed on six individual colonies from each isolate using primers P12 and P80. Of the three cultures, two had six identical PCR profiles; these isolates were designated RU1302 and RU1405.

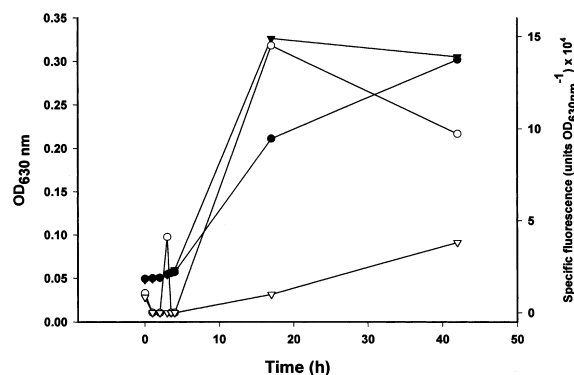


Fig. 2. Induction profile of *thiE* *in vitro* in broth cultures in the absence/presence of additional thiamine. Cultures were grown on AMS minimal medium in the presence of thiamine overnight, washed and resuspended in medium with and without thiamine at time zero. Circles, minus thiamine; triangles, plus thiamine. Open symbols are the fluorescence output in arbitrary units, as detected by a Biolumin 960 fluorescence plate reader, per unit OD₆₃₀, and closed symbols are the OD₆₃₀.

These were reinoculated from purified isolates into minimal medium and the pea rhizosphere. They consistently fluoresced when harvested from root extract but not from broth or agar. The cloned DNA was sequenced from the two isolates using pOT reverse and pOT forward primers (Table 1). The insert DNA in plasmid pRU504 (strain RU1302) has moderate identity (39% over 90 residues) to the 5' end of *thiE* (thiamine phosphate pyrophosphorylase) and is downstream of *thiM* (hydroxythiazole kinase). The insert DNA in plasmid pRU659 (strain RU1405) has high identity (75% over 56 residues) to cyclic β -(1,2)-glucan synthase (*ndvB*) (Table 1).

Thiamine phosphate pyrophosphorylase is an enzyme involved in the final stages of thiamine biosynthesis, and its induction indicated that thiamine may be limiting in the rhizosphere environment. Cyclic β -glucans are required during plant infection and are almost exclusively found in members of the Rhizobiaceae family (Breedveld and Miller, 1994). To determine whether induction of these promoters was a consequence of biotic or abiotic factors, the two strains were inoculated into vermiculite alone, vermiculite containing a pea plant and vermiculite after removal of the pea plant. After harvesting, fluorescence of the strains was observed microscopically. The *ndvB* fusion fluoresced after exposure to vermiculite alone, indicating that it responds to an abiotic factor such as water stress. However, the *thiE* fusion was only expressed when inoculated onto a pea plant or in vermiculite in which a pea plant had been grown. This suggests that the induction may require a soluble plant factor(s).

To investigate the possible causes of induction of *ndvB*, strain RU1405 was tested for osmotic regulation *in vitro* by growing them in AMS minimal medium with glucose as the carbon source and adding 0, 49, 98 or 190 mM NaCl. They were also grown on agar plates containing AMS minimal medium with glucose. There was no increase in expression above that seen in constitutively expressed and unexpressed controls under any conditions (data not shown). This indicates that the *ndvB* promoter was not induced as a consequence of changes in osmotic pressure in the environment or that sufficient hypo/hyperosmotic shock was not provided *in vitro* by growth on AMS liquid medium. Previously, induction of *ndvB* in *S. meliloti* has not been induced by hypo-osmotic shock under conditions in which *ndvB* mutants were impaired for growth (Zorreguieta *et al.*, 1990; Ingram-Smith and Miller, 1998). Transcription of *ndvB* was considered to be constitutive regardless of growth medium osmolarity. It is possible that factors other than osmolarity are responsible for the induction observed here, and this raises the question of what role NdvB may have other than in osmoregulation and plant infection.

Induction of enzymes involved in thiamine biosynthesis

indicates that thiamine may be limiting in the environment. However, like many *R. leguminosarum* strains, strain 3841 is a thiamine auxotroph in laboratory culture. In *Mesorhizobium loti*, the ability to grow in the absence of thiamine is determined by the acquisition of a large chromosomal symbiotic island (Sullivan and Ronson, 1998). Thiamine biosynthetic enzymes may therefore not be induced to levels able to provide sufficient thiamine for extensive population growth of strain 3841. Furthermore, the requirement for a plant factor for induction indicates a more complex process. To test whether the absence of thiamine alone could induce *thiE* expression, induction was investigated *in vitro*. As strain RU1302 cannot grow in the absence of added thiamine, a culture was pregrown on glucose minimal medium with thiamine, then transferred to minimal medium without thiamine. Induction of *thiE* only occurred in the absence of added thiamine, suggesting that induction requires thiamine limitation (Fig. 2). Growth of the culture presumably occurred as a result of the use of residual thiamine carried over from the inoculum. Why then did *thiE* induction fail to occur in cells when placed in vermiculite alone? Perhaps, in pea-associated vermiculite, the plant provided factors for growth, whereas in vermiculite alone, the inoculum merely persisted and induction did not occur. It is possible that *R. leguminosarum* represses thiamine biosynthesis in the absence of other factors required for growth. However, this does not explain the auxotrophic behaviour *in vitro*, where induction of *thiE* does not lead to sufficient thiamine synthesis to support growth. It is interesting that *Rhizobium etli* is a thiamine prototroph and encodes *thiE* on the pB symbiotic plasmid (Miranda-Rios *et al.*, 1997). Curing of pB results in thiamine auxotrophy, and prototrophy was restored by reintroducing *thiCOGE* alone. However, the *R. etli thiE* gene has modest similarity to our sequence (42% over 40 residues). The lack of homology between *thiE* from *R. etli* and strain 3841 may indicate different roles for *thiE* in the two species. Alternatively, there may be more than one copy of the gene in *R. etli*. Further investigations are required to investigate the function of *thiE* in *R. leguminosarum* in the rhizosphere.

Conclusions

We have used DFI-OT to identify temporal gene induction in the rhizosphere, but it can also be used to measure both temporal and spatial gene expression *in situ* in whole tissues or in sectioned samples contained in microchambers. Low-power, solid-state lasers are now available for the construction of optical traps, and a few complete systems are now available commercially. The spatial information given using a microscope in combination with the ability to isolate a specific cell make this a unique tool

in identifying gene expression. However, the specificity and precision of the technique can be time consuming relative to FACS. High-throughput work may be more rapid using FACS combined with Gfp variants better suited for fluorescein isothiocyanate (FITC) filters. We therefore cloned *egfp* into pOT to enable these studies to be performed. However, in our experience, cell size is crucial to FACS and, although larger cells such as *E. coli* can be sorted, we have had limited success in sorting *R. leguminosarum*. The main objective of designing DFI-OT was to enable the identification and isolation of single cells showing specific gene expression from a mixed population. It has succeeded in these objectives and allowed the identification of a number of unique ORFs as well as genes of known function. We believe that the system provides a powerful tool for investigations of environmentally or stress-regulated gene expression.

Experimental procedures

Bacterial strains, plasmids and oligonucleotides

Bacterial strains and plasmids are described in Table 2. Primers used were: P12, GGCTTTACTAAGCTGATCCGGT GG; P13, GGGGATCCGGTGATTGATTGAGCA; P69, GAG AGAGAAGTGTGGAGGAAGAAAAAATGAGTAAAGGAGA AGAAC; P70, ACCGACTAGTAGGCCTATTATT; P79, CCA TTACCTGTCCACACAATCTGCCC; P80, GGGCAGATTGT GTGGACAGGTAATGG; P81, CGATTAATTAAGTCGACAT CTAGAGTTTAAACTTTAATTAAGCCCGGGCTGCA; P82, GCCCGGGCTTAATTAAGTTTAAACTCTAGATGTGCGACT TAATTAAT; P86, GCTTCGCAACGTTCAAATCCGC; P211, GAGAGAGAAGTGTGGAGGAAGAAAAAATGGTGAGCAA GGGCGAGGA; P212, GAGAGAGAGAGCTCTTACTTGTAC AGCTCGTCCA; P246, CTAGGTTAACGCATGCGGGCCCG GTACCAGGCCTAGATAGATAGAGAGAGAGAGAGA; pOT-forward, GCTTTATGCTTGTAACCG; pOT-reverse, TACTC ATTTTTCTCTCCTCCACTAG.

Table 2. Bacterial strains and plasmids.

Strain	Relevant properties	Reference
<i>E. coli</i>		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZDM15</i>) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Hanahan (1983)
XL2-blue	<i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>hsdR17</i> <i>supE44</i> <i>relA1</i> <i>lac</i> [<i>F'</i> <i>proAB</i> <i>lac</i> ^R Δ <i>M15</i> <i>Tn10</i> (Tet ^r) <i>Amy Cam</i> ^r]	Stratagene
<i>R. leguminosarum</i>		
3841	Spontaneous streptomycin-resistant derivative of <i>R. leguminosarum</i> bv. <i>viciae</i> strain 300	Johnston and Beringer (1975); Glenn <i>et al.</i> (1980)
<i>S. meliloti</i>		
Rm1021	Streptomycin derivative of SU47	Meade <i>et al.</i> (1982)
RmH406	Rm1021 <i>phoB3::Tn5-132</i>	Bardin and Finan (1998)
Plasmids		
pBBR1-MCS-1	Broad-host-range cloning vector, Km ^R	Kovach <i>et al.</i> (1994)
pBBR1-MCS-5	Broad-host-range cloning vector, Gm ^R	Kovach <i>et al.</i> (1995)
pCR2.1-TOPO	Cloning vector for PCR products, Am ^R Km ^R	Invitrogen
pEGFP-1	Source of <i>egfp</i> , Am ^R	Clontech Laboratories
pGEM-T	Cloning vector for PCR products, Am ^R	Promega
pGFPuv	Source of <i>gfpuv</i> , Am ^R	Clontech Laboratories
pHP45 Ω	Source of omega interposon St ^R Sp ^R	Prentki and Krisch (1984)
pKK232-8	Source of <i>rrnB</i> transcriptional terminator	Pharmacia
pOTK	Derivative of pBBR1-MCS used for cloning purposes, Sp ^R	This work
pOT1	Promoter probe vector based on pBBR1-MCS-5 replicon. Contains promoterless <i>gfpuv</i> and MCS between two transcriptional terminators. Gm ^R	This work
pOT1e	As for pOT1 but with <i>egfp</i> instead of <i>gfpuv</i>	This work
pOT2	As for pOT1 but with modified polylinker	This work
pRK2013	ColE1 replicon with RK2 <i>tra</i> genes. Used for mobilizing incP and incQ plasmids, Km ^R	This work
pRU496	pOT1 derivative isolated from RU1182	This work
pRU497	pOT1 derivative isolated from RU1187	This work
pRU498	pOT1 derivative isolated from RU1188, <i>metX::gfp</i>	This work
pRU500	pOT1 derivative isolated from RU1184, <i>dimethyltransferase::gfp</i>	This work
pRU504	pOT1 derivative isolated from RU1302, <i>thiE::gfp</i>	This work
pRU514	pOT1 derivative isolated from RU1541	This work
pRU517	pOT1 derivative isolated from RU1203	This work
pRU528	pOT1 derivative isolated from RU1215, <i>poth::gfp</i>	This work
pRU529	pOT1 derivative isolated from RU1216, <i>phytochrome::gfp</i>	This work
pRU530	pOT1 derivative isolated from RU1183, <i>fixN::gfp</i>	This work
pRU582	pOT1 derivative isolated from RU1234, <i>phoC::gfp</i>	This work
pRU583	pOT1 derivative isolated from RU1235, <i>phoA::gfp</i>	This work
pRU584	pOT1 derivative isolated from RU1242, <i>iron-binding protein::gfp</i>	This work
pRU604	pOT1 derivative isolated from RU1535, <i>xyl::gfp</i>	This work
pRU659	pOT1 derivative isolated from RU1405, <i>ndvB::gfp</i>	This work
pNEB193	pUC-derived vector with <i>PacI</i> in MCS suitable for cloning <i>PacI</i> fragments from pOT	New England Biolabs

Growth conditions

Rhizobium leguminosarum was grown at 27°C on acid minimal salts (AMS) (Brown and Dilworth, 1975) with the following modifications: potassium phosphate (0.5 mM), MgSO₄ (2 mM), CaCl₂ (0.17 mM) and MOPS buffer (20 mM, pH 7.0). Minimal medium was supplemented with glucose (10 mM) or succinate (10 mM) and ammonia (10 mM). *E. coli* was grown at 37°C in Luria–Bertani broth (LB). Antibiotics were added as required at the following concentrations unless stated otherwise; gentamicin (20 µg ml⁻¹ for *R. leguminosarum* and 10 µg ml⁻¹ for *E. coli*), streptomycin (250 µg ml⁻¹), nystatin (50 µg ml⁻¹ used as a suspension) and trimethoprim (5 µg ml⁻¹). Transconjugants were selected on AMA supplemented with glucose, ammonia, gentamicin, streptomycin, nystatin and trimethoprim. Trimethoprim and nystatin were added to prevent contamination during storage of plates. For library screening, *R. leguminosarum* strains were grown in 96-well, flat-bottom microtitre plates (Iwaki) in AMS (200 µl) containing glucose, ammonia, gentamicin and streptomycin. Plates were incubated at 27°C and 125 r.p.m. in a custom-built holder mounted on a gyrotary shaker (New Brunswick).

Genetics and molecular biology

Most techniques were as described previously (Sambrook *et al.*, 1989; Poole *et al.*, 1994a). Libraries were conjugated into strain 3841 as described previously (Poole *et al.*, 1994a). To reduce the problem of clonal propagation, the donor was inoculated directly from frozen stock (–80°C) into LB and grown for 4.5 h. The conjugation incubation was reduced to 9 h to limit the number of replication cycles after mating. These times had been determined as the minimum time to give reasonable mating frequencies while minimizing the number of siblings in the final library. To achieve high transformation frequencies for the library construction, super-competent *E. coli* XL2 (Stratagene) were used according to the manufacturer's instructions. Colony and plasmid PCR were performed using Bio-X-Act polymerase (Bioline) on an Omnigene thermal cycler (Hybaid).

Construction of pOT vectors

The *gfpuv* gene was PCR amplified from pGfp-UV using primers P69 and P70 and cloned into pGEM-T. P70 included a *SpeI* site present 3' to the *gfpuv* sequence. To generate a *gfpuv SpeI* cassette, P69 (GAGAGAGA ACTAGTGGAGGAAGAAAAATGAGTAAAGGAGAAGAAC) also contained a *SpeI* recognition site (ACTAGT) 5' to a bacterial ribosome binding site (GGAGGA). The latter was required to optimize fluorescence, as the existing site in *gfpuv* was not suitable. In the construction of *gfpuv*, a *SalI* site had been incorporated into the gene (Cramer *et al.*, 1995). As the library cloning strategy required a single *SalI* site for cloning of partially infilled *Sau3A* DNA fragments, the *SalI* site in *gfpuv* was removed by site-directed mutagenesis using the Quick change kit (Stratagene) and primers P79 and P80. This mutagenesis restored the sequence to that of wild-type Gfp at this location and had no effect on the fluorescence properties of Gfp-UV. Restriction enzyme digestion and DNA sequencing

confirmed this. The *rrnB* transcriptional terminator from pKK232-8 (Pharmacia) was cloned as an *EcoRI* fragment 3' to *gfpuv* in pGEM-T, and the orientation was determined by PCR screening using primers P69 and P86. The Ω transcriptional terminator from pHP45 Ω (Prentki and Krisch, 1984) was isolated by PCR amplification using P12 and P13, cloned in pGEM-T and subsequently as a *PstI*–*Apal* fragment into a pBBR1MCS derivative (Kovach *et al.*, 1994), pOTK (this laboratory). The *gfpuv* gene and the *rrnB* transcriptional terminator were cloned as a *SpeI* cassette 3' to the Ω terminator, and the orientation was determined. The *gfpuv* gene and its associated transcriptional terminators were partially digested with *NcoI*–*StuI* (*gfpuv* contains an internal *NcoI* site) and cloned as a 1.2 kb cassette into *NcoI*–*SspI*-digested pBBR-1-MCS-5. A multiple cloning site linker was constructed using two oligonucleotides, P81 and P82. To orient and clone the linker into the MCS of pBBR1MCS5, P81 and P82 were provided with complementary overhanging ends for *Clal* and *PstI* respectively. All components of the final construct not present in pBBR1-MCS-5 were sequenced, and the plasmid was designated pOT1 (EMBL AJ131020, see Fig. 1).

The *egfp* gene was also cloned into pOT1 as an alternative to *gfpuv*. To achieve this, *egfp* was PCR amplified from pEGfp-1 (Clontech) with primers P211 and P212, and this was cloned into Invitrogen's T-overhang vector pCR2.1-TOPO. The *egfp* gene was swapped for *gfpuv* as a *SpeI*–*SacI* cassette in the pOT1 vector, creating pOT1e. To improve the polylinker of pOT1 a linker was formed by annealing P246 and P247, which was cloned into the *SpeI* of pOT1, creating pOT2 (EMBL AJ310442).

Construction of genomic libraries

Genomic DNA from strain 3841 was partially digested with *Sau3A*I, the 1–3 kb fragments were isolated from agarose using GeneClean (Bio101, according to the manufacturer's instructions) and the termini partially infilled by Klenow DNA polymerase in the presence of dATP and dGTP. GeneClean was found to be superior to other purification systems because it does not bind small fragments, which can contaminate the 1–3 kb DNA. Plasmid pOT1 was digested with *SalI* and treated with dTTP and dCTP, leaving complementary ends to the partially infilled genomic DNA. DNA for automated DNA sequencing using the ALF Express system (Pharmacia Biotech) was extracted and sequenced as described previously (Allaway *et al.*, 1996), using cy5-labelled pOT reverse and pOT forward primers.

Construction of a combined fluorescence optical trapping microscope

The trapping laser is an infrared Nd YAG laser (Spectron 1064 nM model SL501T). A He Ne laser was mounted coaxially behind the trapping laser to provide a visible red beam for ease of alignment (Fig. 3). A beam-expanding telescope (4×) and an electrical shutter were mounted in line with the emerging laser beam. The telescope expands the laser beam width to fill the back aperture of the objective lens. Lasers were used according to the manufacturer's instructions

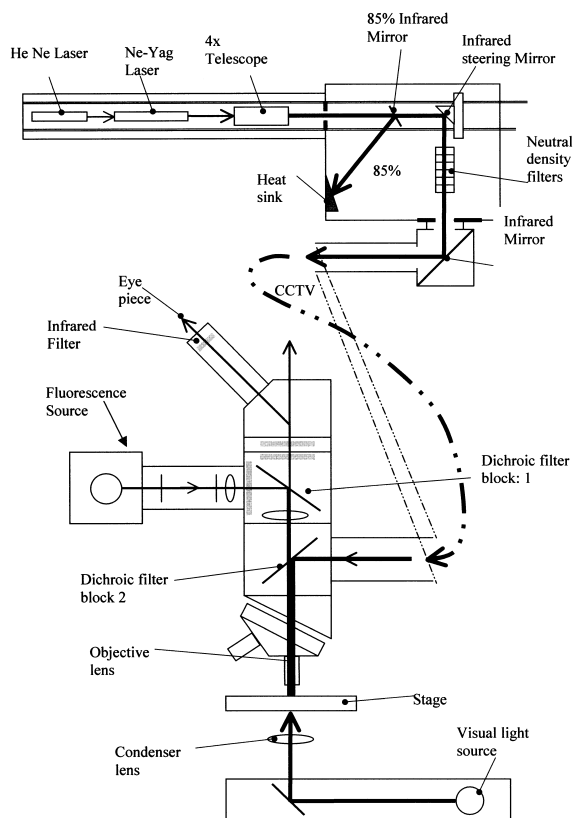


Fig. 3. Schematic of the optical trap showing the laser path (entering from the top), fluorescence UV excitation (entering from the middle) and visual light (entering from below). Note that the top part of the diagram depicting the laser housing and set-up is shown in plan view and the microscope is shown in side view. A detailed description of the construction is in *Experimental procedures* as are details of the dichroic mirrors.

with a power output of between 0.8 and 1.2 W. To trap viable bacterial samples, the power was attenuated using a partial infrared (IR) mirror that reflects 85% of the beam to a heat sink. A custom-made holder for neutral density filters allowed further control of attenuation. Viability was assayed by monitoring the motility of a single bacterium that had been trapped for 1 min and then released. The power used to trap bacteria was 30–45 mW.

The alignment of the laser was carried out empirically. Efficient trapping required the laser to fill the back aperture of the high NA lens, and the beam was centred on the lens so that the trap was in the field of view of the camera. The laser was steered by a series of mirrors and dichroic filters (Fig. 2). The IR beam enters the microscope via a custom-built port and dichroic mounting (Micro Instruments). The laser is reflected vertically down onto the objective lens by dichroic filter 2 (transmits 425–975 nm; Chroma Technology) mounted on a custom-built slider. This enables the IR filter to be removed from the light path if needed. A standard epifluorescence unit was mounted above this to facilitate fluorescence microscopy. This contained dichroic filter block one, which is used for GfpUV (code 11003, Chroma Technology). It contains a 425 nm transmittance (bandwidth 40 nm) exciter filter, a 475 nm longpass emitter filter and a

460 nm longpass dichroic. This is housed in a Nikon filter block on a three-way slider, enabling alternative blocks to be inserted. Standard-phase contrast lenses were used with a 100 \times oil immersion lens (numeric aperture 1.25; Nikon) for trapping. Sterile medium, containing Ficoll (7.5% v/v) to inhibit motility, was drawn by capillary action to three-quarters fill an optically flat microcapillary tube of 0.1 mm path length (Camlab). The bacterial sample was diluted to provide a reasonable number of cells in the field of view and drawn into the remaining quarter of the capillary. The capillary was sealed with Vaseline to reduce cell streaming. Forceps, scalpels and glass slides were flamed with ethanol and the microscope objective wiped with ethanol to maintain sterility. A microscope stage with micrometre controls was used to allow the fine control of the movement of trapped cells. Once a cell was trapped, the stage was moved, and the single cell was moved to sterile medium. The stage was manipulated in order to move the dense gradient of cells away from the trapped cell until it was visually confirmed to be isolated. Immersion oil was removed from the microcapillary slide by swabbing in ethanol, and a scalpel was used to cut away the section containing the isolated bacterium. The section was placed into broth (10 ml) with appropriate antibiotics, incubated under normal growth conditions and then streaked to allow the isolation of single colonies.

Rhizosphere studies

Pea seeds were sterilized by immersion in ethanol (95%) for 30 s, then 2% sodium hypochlorite for 5 min and washed three times in sterile water. These were then germinated in sterile vermiculite and N-free rooting solution (Poole *et al.*, 1994b) and planted in 20 ml of sterile vermiculite. Once the shoot had emerged, 10^5 cfu of bacteria containing various pOT libraries, previously washed and resuspended in sterile water, were inoculated onto each plant. After 7–10 days, individual roots and associated vermiculite were ground in N-free rooting solution (20 ml) using a sterile pestle and mortar. The suspension was filtered through Whatman filter paper, and the filtrate (8 ml) was centrifuged (8500 r.p.m. for 30 s). The supernatant was centrifuged (13 000 r.p.m. for 5 min) to collect bacteria, which were resuspended in N-free rooting solution containing Ficoll (7.5% v/v) for microscopic observation and optical trapping.

To characterize the possible cause of induction of the rhizosphere isolates (*thiE* and *ndvB*), cultures grown in AMS were inoculated (10^5 cfu or 10^8 cfu) into vermiculite, vermiculite containing a pea seedling and vermiculite after carefully removing the pea seedling and all root. Bacteria were harvested at 2 day intervals up to 10 days and observed under the microscope for fluorescence.

Fluorescent observations

Observations of colonies expressing Gfp-UV were made using a transilluminator (UVP model TL-33E) fitted with 365 nm excitation bulbs and a long-wavelength emission filter (420 nm). Observations using a Nikon Optiphot epifluorescence microscope were with standard phase-contrast lenses. Quantitative fluorescence of Gfp-UV in cultures

grown in microtitre plates was determined with a Biolumin 960 plate reader (Molecular Dynamics), using a 405 nm (10 nm bandwidth) excitation filter and a 505 nm emission filter (10 nm bandwidth). For eGfp-1, the excitation filter was changed to 485 nm (10 nm bandwidth). Cell optical density was measured at 630 nm. Fluorescence was linear with OD₆₃₀ over a wide OD range and enabled a simple quantitative relative fluorescence value to be determined (fluorescence emission at 505/OD₆₃₀). Strain RU1159 fluoresced at the threshold of visibility on the transilluminator and under the microscope, so this was inoculated on each microtitre plate as a fluorescence standard control.

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