

# Improved *gfp* and *inaZ* Broad-Host-Range Promoter-Probe Vectors

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**A new set of broad-host-range promoter-probe vectors has been constructed. One subset contains the pVS1 and p15a replicons and confers resistance to either gentamicin or kanamycin. The other set contains the broad-host-range replicon from pBBR1 and confers resistance to kanamycin, tetracycline, ampicillin, or spectinomycin/streptomycin. Both plasmid sets are highly stable and are maintained without selection for more than 30 generations in several bacterial taxa. Each plasmid contains a promoter-probe cassette that consists of a multicloning site, containing several unique restriction sites, and *gfp* or *inaZ* as a reporter gene. The cassette is bound by transcriptional terminators to permit the insertion of strong promoters and to insulate the cassette from external transcription enabling the detection of weak or moderate promoters. The vector suite was augmented with derivatives of the kanamycin-resistant *gfp* promoter-probe plasmids that encode Gfp variants with different half-life times.**

*Additional keywords:* fluorescence, gene expression, ice nucleation, transcriptional fusion.

Transcriptional fusions are important tools in understanding gene expression and gene regulation. The construction of such fusions is greatly facilitated by the use of promoter-probe vectors. These vectors have a common motif in which a promoterless reporter gene, encoding an easily assayable protein, is present downstream of one or more restriction sites. Known promoter sequences or uncharacterized segments of genomic DNA can be ligated into these restriction sites, and the expression of the reporter gene can then be quantified under various conditions. In order to be of the greatest use, promoter-probe vectors should (i) be functional in as many taxa as possible, (ii) show a high degree of sensitivity to detect promoters that

are of weak to moderate strength, and (iii) be stable enough to be used in vivo without antibiotic selection.

Most of the early promoter-probe vectors that were constructed used the *lacZ* reporter gene and contained either the ColE1 or p15a origin of replication (Casadaban and Cohen 1980; Silhavy and Beckwith 1985; Simons et al. 1987). These vectors, while suitable for use in *Escherichia coli*, either could not be maintained in many other taxa or would not be appropriate in organisms or hosts with native  $\beta$ -galactosidase activity. The ability to construct transcriptional fusions in organisms other than *E. coli* is increasingly important; therefore, in order for promoter-probe vectors to be more versatile, they need to contain broad-host-range origins of replication. Several such vectors have been described (Diaz and Garcia 1990; Konyecsni and Deretic 1988; Ronald et al. 1990). Additionally, many organisms are naturally resistant to varying levels of one or more antibiotics (Nikaido and Vaara 1995). Therefore, a set of promoter-probe vectors should also contain a wide variety of antibiotic resistance genes.

Quantification of weak to moderate transcriptional signals in many promoter-probe plasmids is often confounded by transcription that originates upstream of the promoter fusion. This high background level of reporter gene expression eliminates a large subset of promoters from detection by these plasmids. Some promoter-probe vectors have been described in which interference by read-through transcription is largely reduced by the addition of one or more transcriptional terminators upstream of the multicloning site (MCS) (Simons et al. 1987). Although elimination of upstream transcription increases the overall sensitivity of the vector, it is also important to consider the sensitivity of the reporter gene. The level of expression of some reporter genes that have been fused to weakly transcribed promoters may fall below the level of detectability. Some reporter genes, such as *inaZ*, which encodes a bacterial ice nucleation protein, are substantially more sensitive than *lacZ* or *gfp* (W. G. Miller, M. T. Brandl, B. Quiñones, and S. E. Lindow, *unpublished data*). The sensitivity of the reporter gene is also an important consideration when a low-copy-number plasmid, such as a broad-host-range vector, is used.

In this paper, we describe the construction of a set of promoter-probe vectors that combines all of the important features listed above. These promoter-probe vectors contain a common cassette, bound by one or more transcriptional terminators, that consists of an MCS upstream of a promoterless *gfp* or *inaZ* reporter gene. In addition, this set of vectors contains two different broad-host-range origins of replication and

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five antibiotic resistance genes and is readily maintained in several different taxa without antibiotic selection.

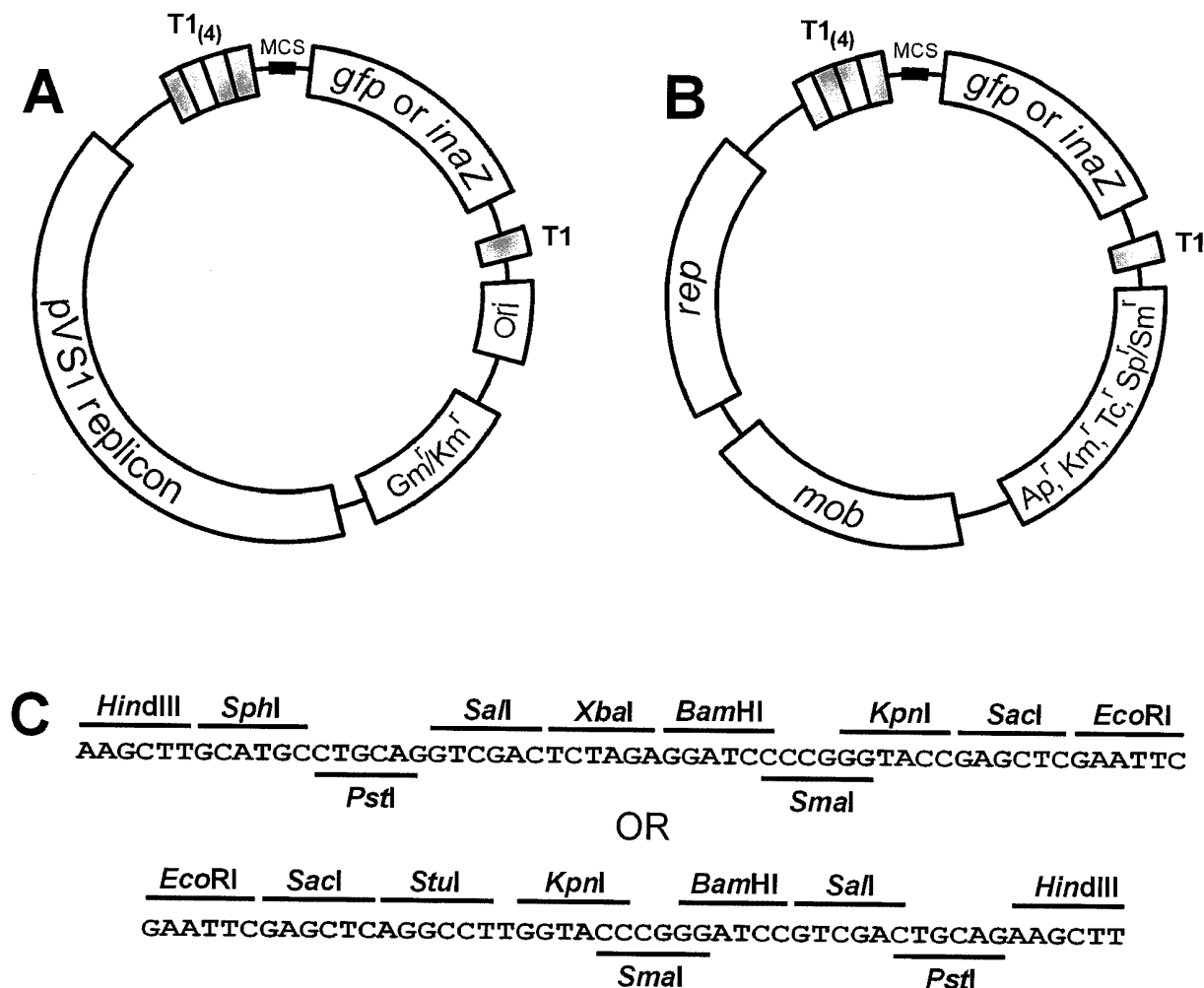
## RESULTS AND DISCUSSION

### Construction of the promoter-probe vectors.

We constructed six sets of promoter-probe plasmids. Two sets contain the pVS1 and p15a replicons, and four sets contain the broad-host-range replicon from pBBR1 (Antoine and Loch 1992) (Fig. 1; Table 1). All members of each set share a common plasmid backbone but contain one of four different

promoter-probe cassettes. Two cassettes in each set use *gfp*, which encodes green fluorescent protein, as the reporter; the other two use the *inaZ* ice nucleation gene. Additionally, in each reporter gene pair, the order of restriction enzymes in the polylinker is either *Hind*III to *Eco*RI (e.g., pPROBE-GT) or *Eco*RI to *Hind*III (e.g., pPROBE-GT').

The first step in the design of the promoter-probe vectors was the construction of a set of reporter gene cassettes. Basically, these cassettes consist of a reporter gene downstream of an MCS, flanked by strong transcriptional terminators and bound on both ends by *Not*I restriction sites. Four different



**Fig. 1.** Promoter-probe vectors. **A**, The pVS1/p15a-derived plasmids. These plasmids contain a region from pVS1 (indicated by 'pVS1 replicon') that contains the broad-host-range *oriV* origin of replication and the *sta* segment for segregation stability (Heeb et al. 2000; van der Bij et al. 1996). In addition, these plasmids contain the *ori* region of pACYC184 ('ori') that encompasses both the p15a origin of transfer, allowing pRK2013- or pRK2073-mediated mobilization of these vectors, and the p15a origin of replication for maintenance in *Escherichia coli*. They also contain the gentamicin resistance gene (*aacC1*) from Tn1696 (Hirsch et al. 1986) or the kanamycin resistance gene (*nptII*) from Tn5. **B**, The pBBR1-derived plasmids. Based on plasmid pBBR1 from *Bordetella bronchiseptica*, these plasmids harbor genes required for replication (*rep*) and mobilization (*mob*) (Antoine and Loch 1992). Like the pVS1-p15a derivatives, these vectors are also mobilizable by conjugation using helper plasmid pRK2013 or pRK2073. These plasmids also contain one of four antibiotic resistance genes: the ampicillin resistance gene (*bla*) from pBR322, the kanamycin resistance gene from Tn*phoA* (Manoil and Beckwith 1985), the tetracycline resistance gene from pWTT2081, or the omega fragment (Prentki and Krisch 1984) which contains the gene encoding resistance to both spectinomycin and streptomycin. Both types of promoter-probe vectors (**A** and **B**) contain a common cassette that has (i) four tandem copies of the T1 terminator (T1<sub>(4)</sub>; shaded boxes) from the *E. coli* *rrnB1* operon (Brosius et al. 1981), (ii) a multicloning site (solid box) containing either **C**, (top) the pUC18 polylinker (*Hind*III → *Eco*RI) or **C**, (bottom) a modified pUC19 polylinker (*Eco*RI → *Hind*III), in which the *Sph*I and *Xba*I sites have been deleted and a *Stu*I site inserted, (iii) the *gfp* or *inaZ* reporter genes (including optimally placed ribosome binding sites: a synthetic one for *gfp* [Miller and Lindow 1997] and the native one for *inaZ*), and (iv) a single *rrnB* T1 terminator (T1; shaded box). In the pPROBE-GT, -GT', -GI, or -GI' vectors, the *Not*I sites are still present at each end of the cassette; they have been disrupted in the other promoter-probe vectors.

cassettes were constructed: two of the cassettes contain the *gfp* reporter gene and the other two, the *inaZ* gene. In addition, two of the cassettes contained, as the MCS, the pUC18 polylinker region. The MCS in the other two cassettes was constructed by replacing the pUC18 polylinker with a linker in which the restriction sites were arranged in reverse order, the *SphI* and *XbaI* sites were removed, and an additional site for a blunt-end restriction enzyme, *StuI*, was inserted. This allows for the construction of fusions in which fragments with differently restricted ends can be ligated in either orientation.

The transcriptional terminator that we chose was the T1 terminator from the *E. coli* *rrmB1* operon (Brosius et al. 1981). This terminator, when placed upstream of the MCS, reduces the background level of expression caused by external transcription (Simons et al. 1987). One copy of the terminator can reduce background expression by 94%; four tandem copies lower the background an additional twofold (97% inhibition), thereby increasing the sensitivity of the vector (Simons et al. 1987). Some strong promoters can only be cloned if a transcriptional terminator is present downstream (Brosius 1984; Gentz et al. 1981); therefore, a single T1 terminator was placed at the 3' end of the cassette.

The first set of promoter-probe vectors contains the broad-host-range pVS1 replicon and either a gentamicin or kanamycin resistance gene (Fig. 1A). These vectors also contain the p15a origin of replication from pACYC184 (*ori*) (Fig. 1A). The presence of four *NotI* sites in the pVS1 replicon made a simple insertion of the *NotI*-ended promoter-probe cassette difficult. Therefore, the *Km<sup>r</sup>* and *Gm<sup>r</sup>* vectors were constructed as follows. The *Km<sup>r</sup>* vectors were constructed by first changing the unique *EcoRI* site in the *cat* gene of the pVS1-derived plasmid pVSP6 to a *NotI*-compatible *Bsp120I* site. Insertion of the promoter-probe cassettes into this *Bsp120I* site created the vectors

pPROBE-KT, -KI, -KT', and -KI'. The *Gm<sup>r</sup>* vectors were constructed by first exchanging the *Tc<sup>r</sup>* gene of pACYC184 with the *aacC1* *Gm<sup>r</sup>* gene (described below) from the transposon Tn5-B22 (Simon et al. 1989). Next, the unique *EcoRI* site in the *cat* gene was changed to a *NotI* site. Finally, the promoter-probe cassettes were ligated into the *NotI* site and a *BamHI*-ended restriction fragment containing the pVS1 replicon was inserted into a unique *BclI* site on the vector to create the plasmids pPROBE-GT, -GI, -GT', and -GI'.

Although the *Gm<sup>r</sup>* and *Km<sup>r</sup>* plasmids were stable without antibiotic selection and exhibited low background levels of expression in multiple taxa, we felt that a larger repertoire of antibiotic resistance was important in order to increase the versatility of this set of promoter-probe plasmids. Recently, a set of broad-host-range cloning vectors derived from the *Bordetella bronchiseptica* plasmid pBBR1 (Antoine and Loch 1992) was described (Kovach et al. 1994, 1995). These plasmids, pBBR1MCS, pBBR1MCS-2, pBBR1MCS-3, pBBR1MCS-4, and pBBR1MCS-5, contain a *lacZα* fragment from pBluescript II-KS and confer resistance to Cm, Km, Tc, Ap, and Gm, respectively. Plasmids containing the pBBR1 replicon are compatible with broad-host-range IncP, IncW, and IncQ group plasmids (Antoine and Loch 1992). Additionally, these plasmids have been shown to be highly stable and replicate in multiple, diverse species (Kovach et al. 1995). We first removed the pBluescript MCS in pBBR1MCS-2 and pBBR1MCS-4 and replaced it with a *Bsp120I* linker, creating the plasmids pKSB2 and pKSB4, respectively. The four promoter-probe cassettes were inserted into pKSB2 to create the plasmids pPROBE-NT, -NI, -NT', and -NI' (Fig. 1B). Similarly, plasmids pPROBE-AT, -AI, -AT', and AI' (Fig. 1B) were constructed by inserting the promoter-probe cassettes into pKSB4. We found the tetracycline resistance gene in pBBR1MCS-3 to be unsuitable, due to the

**Table 1.** Characteristics of the promoter-probe vectors

Plasmid	Resistance	Replicon	Reporter	Multicloning site	Unique restriction sites <sup>a</sup>									
					B	E	H	K	P	Sc	Sa	Sm	St	X
pPROBE-GT	Gm <sup>r</sup>	pVS1/p15a	<i>gfp</i>	<i>HindIII</i> → <i>EcoRI</i>	X	X	X	X	X	X	X	X	X	
pPROBE-GT'	Gm <sup>r</sup>	pVS1/p15a	<i>gfp</i>	<i>EcoRI</i> → <i>HindIII</i>	X	X	X	X	X	X	X	X	X	X
pPROBE-GI	Gm <sup>r</sup>	pVS1/p15a	<i>inaZ</i>	<i>HindIII</i> → <i>EcoRI</i>	X	X	X		X			X		
pPROBE-GI'	Gm <sup>r</sup>	pVS1/p15a	<i>inaZ</i>	<i>EcoRI</i> → <i>HindIII</i>	X	X	X		X			X	X	
pPROBE-KT	Km <sup>r</sup>	pVS1/p15a	<i>gfp</i>	<i>HindIII</i> → <i>EcoRI</i>	X	X	X	X		X				
pPROBE-KT'	Km <sup>r</sup>	pVS1/p15a	<i>gfp</i>	<i>EcoRI</i> → <i>HindIII</i>	X	X	X	X		X			X	
pPROBE-KI	Km <sup>r</sup>	pVS1/p15a	<i>inaZ</i>	<i>HindIII</i> → <i>EcoRI</i>	X	X	X							
pPROBE-KI'	Km <sup>r</sup>	pVS1/p15a	<i>inaZ</i>	<i>EcoRI</i> → <i>HindIII</i>	X	X	X						X	
pPROBE-NT	Km <sup>r</sup>	pBBR1	<i>gfp</i>	<i>HindIII</i> → <i>EcoRI</i>	X	X	X	X		X	X	X		X
pPROBE-NT'	Km <sup>r</sup>	pBBR1	<i>gfp</i>	<i>EcoRI</i> → <i>HindIII</i>	X	X	X	X		X	X	X	X	
pPROBE-NI	Km <sup>r</sup>	pBBR1	<i>inaZ</i>	<i>HindIII</i> → <i>EcoRI</i>	X	X	X					X		X
pPROBE-NI'	Km <sup>r</sup>	pBBR1	<i>inaZ</i>	<i>EcoRI</i> → <i>HindIII</i>	X	X	X					X	X	
pPROBE-AT	Ap <sup>r</sup>	pBBR1	<i>gfp</i>	<i>HindIII</i> → <i>EcoRI</i>	X	X	X	X	X	X	X	X		X
pPROBE-AT'	Ap <sup>r</sup>	pBBR1	<i>gfp</i>	<i>EcoRI</i> → <i>HindIII</i>	X	X	X	X	X	X	X	X	X	
pPROBE-AI	Ap <sup>r</sup>	pBBR1	<i>inaZ</i>	<i>HindIII</i> → <i>EcoRI</i>	X	X	X		X			X		X
pPROBE-AI'	Ap <sup>r</sup>	pBBR1	<i>inaZ</i>	<i>EcoRI</i> → <i>HindIII</i>	X	X	X		X			X	X	
pPROBE-TT	Tc <sup>r</sup>	pBBR1	<i>gfp</i>	<i>HindIII</i> → <i>EcoRI</i>	X	X	X	X		X	X	X		X
pPROBE-TT'	Tc <sup>r</sup>	pBBR1	<i>gfp</i>	<i>EcoRI</i> → <i>HindIII</i>	X	X	X	X		X	X	X	X	
pPROBE-TI	Tc <sup>r</sup>	pBBR1	<i>inaZ</i>	<i>HindIII</i> → <i>EcoRI</i>	X	X	X					X		X
pPROBE-TI'	Tc <sup>r</sup>	pBBR1	<i>inaZ</i>	<i>EcoRI</i> → <i>HindIII</i>	X	X	X					X	X	
pPROBE-OT	Sp <sup>r</sup> /Sm <sup>r</sup>	pBBR1	<i>gfp</i>	<i>HindIII</i> → <i>EcoRI</i>	X	X	X	X	X	X	X	X		X
pPROBE-OT'	Sp <sup>r</sup> /Sm <sup>r</sup>	pBBR1	<i>gfp</i>	<i>EcoRI</i> → <i>HindIII</i>	X	X	X	X	X	X	X	X	X	
pPROBE-OI	Sp <sup>r</sup> /Sm <sup>r</sup>	pBBR1	<i>inaZ</i>	<i>HindIII</i> → <i>EcoRI</i>	X	X	X		X			X		X
pPROBE-OI'	Sp <sup>r</sup> /Sm <sup>r</sup>	pBBR1	<i>inaZ</i>	<i>EcoRI</i> → <i>HindIII</i>	X	X	X		X			X	X	

<sup>a</sup> Restriction enzymes: B = *BamHI*, E = *EcoRI*, H = *HindIII*, K = *KpnI*, P = *PstI*, Sa = *SalI*, Sc = *SacI*, Sm = *SmaI*, St = *StuI*, and X = *XbaI*.

large number of sites for commonly used restriction enzymes (e.g., *EcoRI*, *BamHI*, and *HindIII*) that were present in the coding region. The *Tc<sup>r</sup>* gene in pWTT2081 (van der Bij et al. 1996) does not contain the restriction sites listed above; therefore, in order to construct a *Tc<sup>r</sup>* set of vectors, we first substituted the *Km<sup>r</sup>* gene in pKSB2 with the *Tc<sup>r</sup>* gene from pWTT2081. Insertion of the promoter-probe cassettes into the unique *Bsp120I* site of the *Tc<sup>r</sup>* version of pKSB2 created the plasmids pPROBE-TT, -TI, -TT', and -TI' (Fig. 1B). Finally, another set of promoter-probe plasmids was constructed by replacing the *Km<sup>r</sup>* gene in pKSB2 with the omega fragment, which confers resistance to spectinomycin and streptomycin (Prentski and Krisch 1984), and inserting the promoter-probe cassettes, creating the vectors pPROBE-OT, -OI, -OT', and -OI' (Fig. 1B).

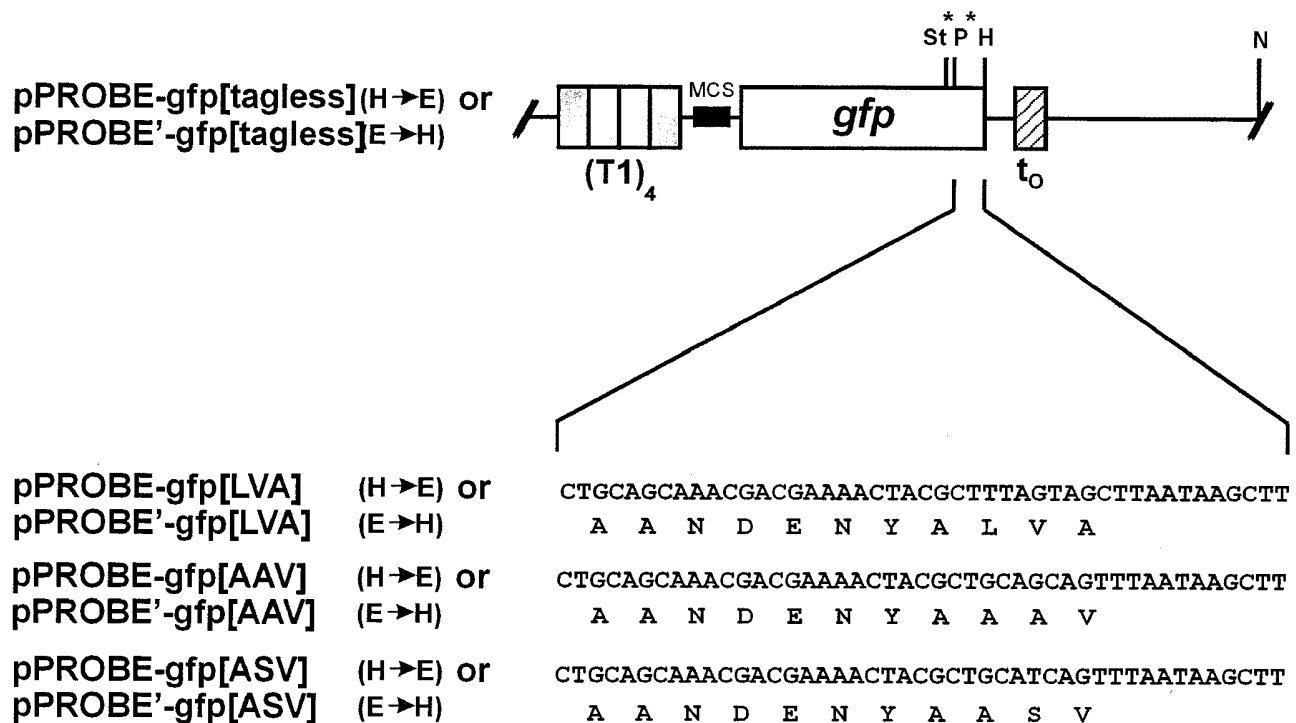
### Construction of *gfp* half-life variant plasmids.

Wild-type Gfp is an extremely stable protein: cells containing this protein show no decrease in fluorescence over at least 24 h (Andersen et al. 1998), a half-life that extends, essentially, over many bacterial generations. Although this stability can be an advantage at times, the long half-life of Gfp can complicate studies of gene regulation in which *gfp* expression decreases over time or under various environmental conditions. Recently, unstable *gfp* variants have been described (Andersen et al. 1998); addition of an AANDENYALAA tag to the C-terminal end of wild-type Gfp reduced the half-life of the protein to approximately 40 min in *E. coli* (Andersen et al. 1998). By altering the final three codons on this tag, Andersen et al. (1998) constructed three different alleles, Gfp[LVA],

Gfp[AAV], and Gfp[ASV] with half-lives of approximately 40, 60, and 110 min, respectively. In order to incorporate these tagged *gfp* alleles into our set of promoter-probe vectors, we replaced the *HpaI-NsiI* fragment from pPROBE-NT and pPROBE-NT' that contains the C-terminal portion of *gfp* and the *rrnB1* T1 terminator with a *HpaI-NotI* fragment from the plasmids pJBA28 (wild-type Gfp), pJBA116 (Gfp[LVA]), pJBA118 (Gfp[AAV]), and pJBA120 (Gfp[ASV]) (Andersen et al. 1998). These half-life variant plasmids were designated pPROBE-*gfp*[tagless], -*gfp*[LVA], -*gfp*[AAV], and -*gfp*[ASV] and pPROBE'-*gfp*[tagless], -*gfp*[LVA], -*gfp*[AAV], and -*gfp*[ASV], respectively (Fig. 2).

### Sensitivity and stability of the promoter-probe vectors.

It is very important for promoter-probe vectors to have as low a basal level of expression as possible in order to detect promoters of weak to moderate strength. The level of reporter gene activity in the absence of a promoter should be as close as possible to the activity of a strain without the plasmid. In order to quantify the background level of *gfp* and *inaZ* expression in the set of plasmids described above, we transformed different pPROBE constructs into the following four strains: *E. coli* DH5 $\alpha$ , *Erwinia herbicola* 299R, *Pseudomonas syringae* pv. *syringae* B728a, and *P. syringae* cit7del1. The fluorescence of cells transformed with pPROBE-GT did not differ from the fluorescence of untransformed strains (Table 2). Likewise, strains transformed with *inaZ* promoter-probe plasmids pPROBE-GI or pPROBE-KI had very low levels of ice nucleation, comparable to plasmidless strains (Table 2). The



**Fig. 2.** *gfp* half-life variants of pPROBE-NT and pPROBE-NT'. The structures of the half-life-variant promoter-probe cassettes are shown (not drawn to scale). pPROBE-*gfp*[tagless] and pPROBE'-*gfp*[tagless] were derived from pPROBE-NT and pPROBE-NT', respectively, and contain wild-type *gfp* alleles. Plasmids pPROBE-*gfp*[LVA], -*gfp*[AAV], and -*gfp*[ASV] were derived from pPROBE-NT. Plasmids pPROBE'-*gfp*[LVA], -*gfp*[AAV], and -*gfp*[ASV] were derived from pPROBE-NT'. The *StuI* and *PstI* restriction sites are present in all but the [tagless] plasmid variants. (H→E) and (E→H) represent the orientation of restriction sites in the polylinker (Table 1; Fig. 1C). (T1)<sub>4</sub> = four tandem T1 terminators from the *Escherichia coli* *rrnB1* operon, t<sub>0</sub> = phage lambda t<sub>0</sub> terminator, H = *HindIII*, N = *NotI*, St = *StuI*, and P = *PstI*.

moderate level of ice nucleation in strain B728a(pPROBE-GI) reflects the native ice nucleating ability of wild-type strain B728A. To verify that these vectors would detect transcriptional signals, a 131-base pair (bp) *HindIII-BamHI* fragment, containing the *nptII* promoter (kan) from Tn5, was cloned into the MCS of pPROBE-GT, pPROBE-GI, and pPROBE-KI, creating pPROBE-GIkan, pPROBE-GIkan, and pPROBE-KIkan, respectively. Cells transformed with these kan-*gfp* or kan-*inaZ* fusions showed a large increase in either fluorescence or ice nucleation (Table 2), indicating that these promoter-probe plasmids are suitable for the construction of transcriptional fusions. The Gfp data suggest that a promoter approximately 35-fold weaker than the moderately strong *nptII* promoter would give a fluorescence signal that approaches the limit of detection; conversely, anything stronger than that should give a signal well above background. InaZ, on the other hand, is a much more sensitive reporter, which allows detection of even weaker promoters; in *E. herbicola* 299R, for example, the number of ice nuclei per cell is almost 10<sup>7</sup> times higher with pPROBE-GIkan than with pPROBE-GI (Table 2). Due to the log-linear relationship between the amount of InaZ protein and the signal it generates (Lindgren et al. 1989), this translates into a more than 3,000-fold higher abundance of InaZ per cell. This then suggests that even a very weak promoter with a 3,000-fold lower transcriptional activity than *nptII* gives a signal well above background level.

For measurements of gene expression in vitro, where antibiotic selection can be maintained over the time course of an experiment, plasmid stability is not a factor. However, in many cases, antibiotics cannot be applied in situ and plasmids must be maintained in the absence of selection. To quantify the stability of the pVS1 and pBBR1 plasmids, we transformed 299R with either pPROBE-GT, pPROBE-GI, or pPROBE-NT and grew the resulting transconjugants in Luria-Bertani (LB) broth without selection for several generations. No plasmid loss was detected after 56 generations in either 299R(pPROBE-GT) or 299R(pPROBE-GI), or after 34 generations in 299R(pPROBE-NT) (data not shown). Hallmann et al. found similar results using pPROBE-GT transconjugants of *Rhizobium etli* grown for more than 80 generations without selection (J. Hallmann, A. Quadt-Hallmann, W. G. Miller, and

S. E. Lindow, unpublished data). Additionally, in experiments with 299R(pPROBE-GTkan), after 34 generations without selection, >99% of the colonies remained fluorescent. A 100,000-fold increase in cell number represents approximately 16 to 17 bacterial generations; therefore, the stability of these plasmids over more than 30 generations without selection far exceeds the requirements for most experiments in situ.

In some strains, especially those that contain a wild-type *recA* allele, recombination events may occur between the T1 terminators. This would involve either recombination between the four tandem upstream terminators, resulting in a decrease or increase in the number of upstream terminators, or recombination between the upstream terminators and the single downstream terminator, resulting in loss of the promoter-probe cassette. Recombination among the upstream terminators would only result, at worst, in a slight increase in basal reporter gene activity, because even one upstream terminator can reduce the background level of expression by 94%. Cells in which recombination between the upstream terminators and the single downstream terminator has occurred are enriched only when the reporter gene product is overexpressed to levels that are lethal to the cell (e.g., with an extremely strong promoter). Therefore, we strongly suggest that recombination events be assessed in each strain for each fusion before quantifying reporter gene activity.

## Conclusions.

This report describes the construction of a set of 24 highly stable, broad-host-range promoter-probe vectors. These plasmids use either *gfp* or *inaZ* as the reporter gene. The construction of transcriptional fusions in these vectors is facilitated by the presence of two different MCSs, each containing numerous unique restriction sites. The presence of four terminators positioned upstream of the MCS confers a low basal level of reporter gene expression, thus enabling the detection of promoters of weak to moderate strength. In addition, this set of promoter-probe vectors contains five different antibiotic resistance genes; therefore, these vectors can be used in taxa with native antibiotic resistance (provided that the strain in question is not resistant to all five antibiotics) or in experiments involving two or more plasmids. Finally, a subset of

**Table 2.** Expression of *gfp* and *inaZ* from different promoter-probe vectors in different hosts

Strain	<i>gfp</i> expression		<i>inaZ</i> expression	
	Plasmid <sup>a</sup>	Fluorescence <sup>b</sup>	Plasmid <sup>a</sup>	Ice nucleation activity <sup>b</sup>
<i>Escherichia coli</i> DH5 $\alpha$	None	6.0	n.a. <sup>c</sup>	n.a.
	pPROBE-GT	6.9	n.a.	n.a.
	pPROBE-GTkan	294	n.a.	n.a.
<i>Erwinia herbicola</i> 299R	None	6.6	None	<8.24
	pPROBE-GT	6.6	pPROBE-GI	<8.07
	pPROBE-GTkan	338	pPROBE-GIkan	-1.08
<i>Pseudomonas syringae</i> B728a	None	6.7	None	-4.24
	pPROBE-GT	6.9	pPROBE-GI	-3.98
	pPROBE-GTkan	243	pPROBE-GIkan	-0.89
<i>Pseudomonas syringae</i> cit7del1	n.a.	n.a.	None	<9.53
	n.a.	n.a.	pPROBE-KI	-8.14
	n.a.	n.a.	pPROBE-KIkan	-3.29

<sup>a</sup> Plasmids pPROBE-GTkan, pPROBE-GIkan, and pPROBE-KIkan contain a 131-base pair *nptII* promoter fragment from Tn5 fused to *gfp* (pPROBE-GTkan) or *inaZ* (pPROBE-GIkan and -KIkan).

<sup>b</sup> Enzyme assays were performed as described in Materials and Methods. Fluorescence is expressed in arbitrary units normalized for 10<sup>9</sup> cells. InaZ activity is expressed as log[ice nuclei per cell]. Each value is the mean of three replicate samples.

<sup>c</sup> n.a. = Not applicable.

plasmids has been constructed in which the wild-type *gfp* allele in two of the vectors described above (i.e., pPROBE-NT and pPROBE-NT') has been replaced with unstable *gfp* variants allowing real-time studies of gene regulation in situ.

#### Nucleotide sequence accession numbers.

As a representative subset of the pBBR1-based vector suite, we have deposited in GenBank the complete nucleotide sequences of all 12 pBBR1-derived vectors conferring resistance to kanamycin. Accession numbers are as follows: pPROBE-NT, AF286453; pPROBE-NT', AF286454; pPROBE-NI, AF286451; pPROBE-NI', AF286452; pPROBE-*gfp*[tagless], AF286455; pPROBE'-*gfp*[tagless], AF286456; pPROBE-*gfp*[LVA], AF286461; pPROBE'-*gfp*[LVA], AF286462; pPROBE-*gfp*[AAV], AF286457; pPROBE'-*gfp*[AAV], AF286458; pPROBE-*gfp*[ASV], AF286459; and pPROBE'-*gfp*[ASV], AF286460. The nucleotide sequences of all other pBBR1-derived plasmids (with the exception of those conferring resistance to tetracycline: the pWTT2081 Tc<sup>r</sup> locus has not been sequenced) can be readily compiled using the above sequences, the cloning steps described in detail below, and the available sequence data for pBBR1MCS-2 (U23751), pBBR1MCS-4 (U25060), and the omega fragment (M60473). Unfortunately, the pVS1-based plasmids could not be considered for submission due to segments with unknown DNA sequences.

## MATERIALS AND METHODS

#### Media, strains, growth conditions, and chemicals.

Bacterial strains were grown either on LB agar or in LB broth. *E. coli* DH5 $\alpha$  was grown at 37°C, whereas *Erwinia herbicola* 299R (Brandl and Lindow 1996), *P. syringae* pv. *syringae* B728a (Loper and Lindow 1987), and *P. syringae* cit7del1 (Lindow 1985) were grown at 24°C. When used, gentamicin (Gm), kanamycin (Km), ampicillin (Ap), tetracycline (Tc), spectinomycin (Sp), streptomycin (Sm), and rifampicin (Rf) were added at final concentrations of 15, 50, 150, 15, 100, 100, and 100  $\mu$ g/ml, respectively. Restriction and DNA-modifying enzymes were purchased from New England Biolabs (Beverly, MA, U.S.A.) or Roche Molecular Biochemicals (Indianapolis, IN, U.S.A.). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). All oligonucleotides were synthesized by Oligos, Etc. (Wilsonville, OR, U.S.A.).

#### Construction of the promoter-probe cassettes.

The *gfp* and *inaZ* promoter-probe cassettes were constructed in three steps. First, *gfp* and *inaZ* were amplified from pGreenTIR (Miller and Lindow 1997) and pUC1813ice (W. G. Miller, M. T. Brandl, B. Quiñones, and S. E. Lindow, unpublished data), respectively, and fused to a single *rrnB1* T1 terminator (Brosius et al. 1981) through a polymerase chain reaction (PCR) overlap extension method (Horton et al. 1990). *gfp* was amplified using oligos T14GFP5' (5' ATTCCTAACTAATAATGATTAACTTTATAAGGAGGAAAAAC 3'; ribosome-binding site underlined) and T1GFP3' (5' GATGCTGGAATTAATTCCTATTTGTATAGTTCATCC 3'), and *inaZ* with oligos T14ICE5' (5' ATTCCTAACTAATAACCCCAAAGGATCTATGAGGATGC 3'; putative ribosome-binding site underlined) and T1ICE3' (5' GATGCTGGAATGCGGGCTACTTTACCTCTATCCAGTC 3'). The T1 ter-

minator was amplified from pRS1197 (W. G. Miller, unpublished data) using oligos T1NOT3' (5' CCGCGGCCGCCCAATTCCTGGCAGTTTATGG 3') and either T1GFP5' (5' CAAATAGAATTAATTCAGGCATCAAATAAAAGAAAGGC 3') or T1ICE5' (5' GTAATAGCCGCATTCGGCCAAATAAACGAAAGGC 3'). All four PCR reactions were carried out for 30 cycles of 1 min at 95°C, 2 min at 50°C, and 3 min at 72°C. A sample of the amplified product from the *gfp* and T1 (using oligo T1GFP5') reactions were added to a second PCR reaction containing oligos T1NOT3' and T14GFP5'. Likewise, a sample of the amplified product from the *inaZ* and T1 reactions (using oligo T1ICE5') was added to a second PCR reaction containing oligos T1NOT3' and T14ICE5'. Both PCR reactions were carried out for 30 cycles of 1.5 min at 95°C, 2 min at 50°C, and 4 min at 72°C. The amplified products were ligated into pCR2.1 (Invitrogen, Carlsbad, CA, U.S.A.) to generate a 5' *EcoRI* site and create the plasmids pCR8 (*gfp*) or pCR7 (*inaZ*).

Second, a plasmid containing four tandem copies of the T1 terminator fused to the pUC18 polylinker was constructed by first altering the plasmid pRS391 (Simons et al. 1987), which contains four T1 terminators flanked on the 5' end by an *EcoRI* site and on the 3' end by a *BamHI* site. In separate reactions, the *EcoRI* and *BamHI* sites were filled in with DNA polymerase (Klenow fragment) and ligated to an 8-bp *NotI* linker and a 10-bp *HindIII* linker (5' GGAAGCTTCC 3'), respectively. The resulting plasmid and pUC18 were then digested with *HindIII* and *ScaI* and ligated together to create the plasmid pNH18.

Finally, the *NotI*-ended promoter-probe cassettes were constructed by (i) digesting pNH18 with *NotI* and *EcoRI*, (ii) digesting pCR7 and pCR8 with *EcoRI* and *NotI*, and (iii) ligating the reporter gene fragments and the T1<sub>(4)</sub>-MCS fragment into *NotI* digested pBluescript KS (Stratagene, La Jolla, CA, U.S.A.) to create the plasmids pNH18/8 (*gfp*) and pNH18/7 (*inaZ*). Both promoter-probe cassettes were verified by extensive restriction digest analysis and DNA sequencing. During the amplification of *inaZ*, a 15-bp deletion occurred: the sequence from the *EcoRI* site to the start codon is therefore 5' GAATTCGGCTTAACCCCAAAGGATCTATGAGGATGCTGT AATG 3'.

#### Construction of the promoter-probe vectors.

To create a Gm<sup>r</sup> backbone for the promoter-probe vectors pPROBE-GT, -GI, -GT', and -GI', pACYC184 was modified in several steps. First, the *aacC1* gene, which confers Gm resistance, was amplified from the transposon Tn5-B22 (Simon et al. 1989) using oligos Gm-5' (5' GGAATCCCAA-TTTACCGAACAACCTCCG 3') and Gm-3' (5' GGAATCCCAAACGATGCTCGCCTTCC 3'). PCR reactions were carried out for 30 cycles of 1 min at 95°C, 2 min at 50°C, and 2 min at 72°C. The amplified product was digested with *EcoRI* and ligated to *EcoRI*-digested pUC1813 (Kay and McPherson 1987) to create pUC1813Gm. pUC1813Gm was digested with *EcoRI*, filled in with DNA polymerase (Klenow fragment), and ligated to pACYC184, which had been digested with *HincII* and *HindIII* and filled in with DNA polymerase (Klenow fragment). This step essentially swapped the Tc<sup>r</sup> gene of pACYC184 with a Gm<sup>r</sup> gene. Next, the unique *EcoRI* site present in the Cm<sup>r</sup> gene was filled in and ligated to an 8-bp *NotI* linker. The two *NotI*-ended promoter-probe cassettes

were then ligated into this *NotI* site to create the plasmids pG7 (*inaZ*) and pG8 (*gfp*). Finally, the broad-host-range pVS1 replicon containing replication and stability functions was inserted into pG7 and pG8 by first ligating an approximately 7.8-kb filled-in *BamHI-SalI* fragment from pWTT2081 (van der Bij et al. 1996) into *SmaI*-digested pUC1813 and then ligating a *BamHI*-ended pVS1 fragment into *BclI*-digested pG7 and pG8 to create pPROBE-GI and pPROBE-GT, respectively.

Additionally, two promoter-probe vectors (pPROBE-GT' and -GI') were created in which the order of the restriction sites in the MCS was reversed. Two linkers were synthesized so that, when annealed together, they would form a double-stranded-MCS adaptor with a 5' *HindIII* compatible end and a 3' *EcoRI* compatible end. Plasmids pPROBE-GT and pPROBE-GI were digested with *HindIII* and *EcoRI* and ligated to the new MCS adaptor, creating pPROBE-GT' and pPROBE-GI', respectively. Insertion of the adaptor disrupted the original *HindIII* and *EcoRI* sites. The MCS adaptor does not contain *XbaI* or *SphI* sites; also, in order to facilitate blunt-ended cloning in situations in which the *SmaI* site is not unique, a *StuI* site was added. This *StuI* site is unique in pPROBE-GT', pPROBE-GI', and all subsequent promoter-probe constructs containing the reverse MCS.

In order to create the promoter-probe vectors pPROBE-KT, -KI, -KT', and -KI', the four *NotI*-ended promoter-probe cassettes were to be inserted into the  $\text{Cm}^r$ ,  $\text{Km}^r$  broad-host-range vector pVSP6 (obtained from William Tucker, DNA Plant Technologies, Oakland, CA, U.S.A.). The presence of four *NotI* sites in the pVS1 replicon of that plasmid made a simple ligation difficult. Therefore, a *Bsp120I* linker (5' AATTGGG-GCCCC 3') was synthesized and annealed to itself to form a double-stranded adaptor with *EcoRI*-compatible ends. This adaptor was then ligated into the unique *EcoRI* site present within the *cat* gene of pVSP6, disrupting this site in the process. This plasmid was digested with *Bsp120I* and ligated to the four *NotI* ended promoter-probe fragments to create pPROBE-KT, -KI, -KT', and -KI'.

An additional set of promoter-probe vectors with a wider variety of antibiotic resistances was constructed by modifying the previously described pBBR1MCS series of broad-host-range vectors (Kovach et al. 1995). The MCS in the plasmids pBBR1MCS-2 and pBBR1MCS-4 was replaced with a unique *Bsp120I* site by a procedure similar to that described above. Two oligos (5' GGGGCCCGTAC 3' and 5' GGGGCCCA-GCT 3') were synthesized and annealed to generate a double-stranded adaptor with *KpnI*- and *SacI*-compatible ends. pBBR1MCS-2 and pBBR1MCS-4 were digested with *KpnI* and *SacI* and ligated to the *Bsp120I* adaptor to create plasmids pKSB2 and pKSB4, respectively. The  $\text{Km}^r$  gene in pKSB2 was replaced by either a  $\text{Tc}^r$  gene or the omega fragment that confers Sp/Sm resistance (Prentki and Krisch 1984). This was accomplished by making use of the unique *Bsp120I* restriction site present on pBluescript KS and the unique *BglIII* site present 3' of the  $\text{Km}^r$  gene on pKSB2. A *HindIII*-ended omega fragment from pUC1318O (E. Clark, unpublished data: constructed by inserting an approximately 2-kb *HindIII*-ended omega fragment [Prentki and Krisch 1984] into the *HindIII* site of pUC1318 [Kay and McPherson 1987]) was filled in with DNA polymerase (Klenow fragment) and ligated to *HincII/SmaI*-digested pBluescript KS. The resulting plasmid was digested with *Bsp120I* and *BamHI* and the approximately

2-kb Sp/Sm<sup>r</sup> fragment was ligated to *Bsp120I/BglIII*-digested pKSB2 to create the plasmid pKSB $\Omega$ . Similarly, an approximately 2.2-kb filled-in *HindIII/SalI* fragment from pWTT2081, which confers Tc resistance, was cloned into pKSB2 via pBluescript KS to create the plasmid pKSB2tet. Finally, the four *NotI*-ended promoter-probe cassettes were cloned into *Bsp120I*-digested plasmids pKSB2, pKSB4, pKSB $\Omega$ , and pKSB2tet to create the plasmids pPROBE-NT, -NI, -NT', -NI', -AT, -AI, -AT', -AI', -OT, -OI, -OT', -OI', -TT, -TI, -TT', and -TI', respectively.

#### ***gfp* half-life-variant plasmids.**

To construct the *gfp* half-life variant plasmids, the *HpaI-NotI* fragment containing the C-terminal portion of *gfp* and the bacteriophage lambda  $t_0$  terminator from plasmid pJBA28, pJBA116, pJBA118, or pJBA120 (Andersen et al. 1998) was first cloned into the plasmid pCR2.1. The *gfp*-terminator fragment was then excised from the pCR2.1 subclones with *HpaI* and *PstI*. Plasmids pPROBE-*gfp*[tagless], pPROBE-*gfp*[LVA], pPROBE-*gfp*[AAV], and pPROBE-*gfp*[ASV] were constructed by replacing the *HpaI-NsiI* fragment of pPROBE-NT, containing the C-terminal portion of *gfp* and the *rrnBI* T1 terminator, with the *HpaI-PstI* fragment. Plasmids pPROBE'-*gfp*[tagless], pPROBE'-*gfp*[LVA], pPROBE'-*gfp*[AAV], and pPROBE'-*gfp*[ASV] were constructed in a similar manner by replacing the *HpaI-NsiI* fragment of pPROBE-NT'.

#### **Plasmid transformation.**

Plasmid DNA was conjugated into *Erwinia herbicola* 299R and the *P. syringae* strains by triparental mating using *E. coli* strain DH5 $\alpha$  as the donor and either DH5 $\alpha$  (pRK2013) (Figurski and Helinski 1979) or DH5 $\alpha$  (pRK2073) (Better and Helinski 1983) as the helper.

#### **Enzyme assays.**

Cells from overnight LB cultures were centrifuged, washed once with 10 mM phosphate buffer, and resuspended in phosphate buffer at a final concentration of approximately  $2 \times 10^9$  cells per ml. Ice nucleation assays were performed as previously described (Lindow 1990) with cells that were grown at 28°C. Cells containing the InaZ protein exhibit a maximum frequency of ice nucleation when cultures are grown in the temperature range of 18 to 24°C (Lindow 1995). Even though the ice nucleation frequency is reduced approximately 100-fold when cells are grown at 28°C (Lindow 1995), the ice nucleation at that temperature is still well above background. Most strains of *E. coli* will grow at 28°C; however, in this experiment, DH5 $\alpha$  did not grow well at this temperature. Therefore, ice nucleation assays were not performed for this strain. For the *gfp* promoter-probe vectors, fluorescence was measured on a Perkin-Elmer LS50B Luminescence Spectrometer (Perkin Elmer Instruments, Norwalk, CT, U.S.A.) at an excitation wavelength of 490 nm, an emission wavelength of 510 nm, and emission/excitation slit widths of 8 nm. Intensity readings are represented by arbitrary units and were normalized to a cell density of  $10^9$  cells per ml.

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