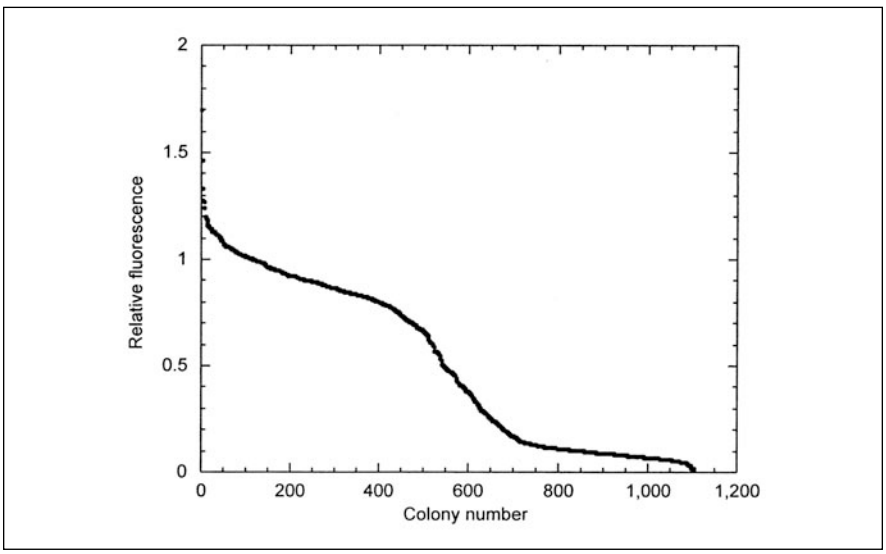

Figure 3. Screening of an error-prone PCR library of GFP. Megaprimers were prepared by error-prone PCR, and whole plasmid PCR was run using the products. A total of 1152 clones was screened for fluorescence at 509 nm (excitation wavelength, 488 nm). Fluorescence level of parent GFP+ was taken as 1.

Advantages of the Present Method

MEGAWHOP has a number of advantages over other methods and has potential applications. First, this approach is quick and simple. This merit is maximized when one attempts to produce random mutagenesis libraries by error-prone PCR. When the backbone plasmid and template plasmid used for random mutagenesis are identical, there is no need to separate the template plasmid and the error-prone PCR products using tedious agarose gel electrophoresis. Second, the quality (i.e., cloning efficiency) of the library is highly independent of the reaction conditions. Therefore, it is possible to create libraries without monitoring intermediate steps, if the number of clones is not so critical. In addition, if one attempts to draw a "fitness landscape" of a random mutant library and to extract certain scientific information (such as mutation frequency and dead/alive ratio), then it is essential that each clone contain a single insert. Third, the procedure enables the DNA fragment to be cloned directly in the vector, independent of restriction enzyme sites. Because of this, one can modify any given gene segment of interest without being limited by the recognition sequence of restriction enzymes. This is particularly useful when modifying flanking sequences of a gene segment is not recommended. For example, one can specifically modify only a mature region of protease without affecting junctions between pro- and mature sequences. All these features of the MEGAWHOP cloning method are ideal for creating random mutagenesis libraries. So far, we have validated the method using megaprimer of 200 bp to 1 kb and template plasmid of 3.2–8 kb. This indicates that the method is quite flexible and useful for most common DNA cloning experiments.

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Broad-Host-Range Mobilizable Suicide Vectors for Promoter Trapping in Gram-Negative Bacteria

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ABSTRACT

Here we report the construction of three different vectors for the identification of bacterial genes induced in vitro and/or in vivo. These plasmids contain kanamycin, gentamicin, or tetracycline resistance genes as selectable markers. A promoterless cat *and an improved GFP (*mut3-gfp*) can be*

used to follow the induction of gene expression by measuring chloramphenicol resistance and fluorescence, respectively.

INTRODUCTION

Although the complete DNA sequences of several microbial genomes are now available, many putative genes remain to be characterized. Comprehensive screening methods for identifying functional classes of genes are needed to convert genome database sequences into meaningful biological information. Functional genomics that include in vivo expression technology (IVET) (17), signature-tagged mutagenesis (8,10), differential fluorescence induction (18), and microarray analyses (9) might assign new functions to sequenced genes and can explain the patterns of gene expression in response to specific environmental stimuli. A particularly important group of bacterial genes contributes to the fitness of a microorganism during interactions with its host. As a consequence, the expression of these genes is enhanced in vivo. IVET-based techniques can be used for the isolation of environmentally induced genes (6). Here we describe a new set of IVET vectors that are transcriptionally coupled to genes that code for chloramphenicol resistance as a selectable marker and a mutant of GFP with enhanced fluorescence (*mut3-gfp*) as a reporter system. In addition to the transcriptional fusion module, each vector carries a kanamycin, tetracycline, or gentamicin resistance cassette, thus expanding the application of the IVET strategy to other Gram-negative bacteria that are naturally resistant to ampicillin.

MATERIALS AND METHODS

Table 1 lists the bacterial strains and plasmids used in this study.

Construction of pMIK, pMIT, and pMIG Vectors

The series of vectors constructed by the ligation of the 900-bp *Sal*I fragment from pIVET8 (11) that contained a promoterless *cat* gene to pKNOCK-Tc, pKNOCK-Gm, and pKNOCK-Km (1) generated the pCat-Tc, pCat-Gm, and pCat-Km vectors, respectively. Next, a 700-bp *Hin*dIII/*Xba*I restriction digest of pKEM (3) that contained the promoterless *mut3-gfp* was purified. The ends of this fragment were filled in with Klenow DNA polymerase and ligated to the end-filled *Xho*I terminus of pCat-Km, pCat-Gm, and pCat-Tc. The

Table 1. Bacterial Strains and Plasmids Used in this Work

Strain/Plasmid	Relevant Properties	Source/Reference
Rhizobium sp. NGR234	Rif^r derivative of wild-type strain NGR234 from *Lablab purpureus*	(5)
E. coli S17-1 λpir	Tp^r,Sm^r *recA*, *thi*, *pro*, *hsdR$^-$M$^+$*, RP4: Mu-Km::Tn7, λpir	(14)
Plasmid		
pKNOCK-Km	OriR6K mobRP4, Km^r	(1)
pKNOCK-Gm	OriR6K mobRP4, Gm^r	(1)
pKNOCK-Tc	OriR6K mobRP4, Tc^r	(1)
pIVET8	Synthetic operon consisting of promoterless *cat* and promoterless *lacZY* genes cloned into the broad-host-range suicide vector pGP704 (Amp^r)	(11)
pKEM	pKEN carrying *mut3-gfp*	(3)
pCat-Km	OriR6K mobRP4 Km^r, promoterless *cat*	This study
pCat-Gm	OriR6K mobRP4 Gm^r, promoterless *cat*	This study
pCat-Tc	OriR6K mobRP4 Tc^r, promoterless *cat*	This study
pMIK	pCat-Km, promoterless *mut3-gfp*	This study
pMIG	pCat-Gm, promoterless *mut3-gfp*	This study
pMIT	pCat-Tc, promoterless *mut3-gfp*	This study
pNBA	pMP220 containing the promoter of *nodABC* genes	(5)

resulting plasmids, pMIK, pMIG, and pMIT (Figure 1) are suicide vectors that contain promoterless *cat* and *mut3-gfp* genes transcribed in tandem. The upstream region 5′ of *cat* and *mut3-gfp* contains *Bam*HI, *Sma*I, and *Cla*I cloning sites, useful for the random cloning of bacterial DNA fragments.

Testing the New Vectors

Rhizobium sp. NGR234 is known to form nitrogen-fixing symbioses with a wide variety of legumes. Phenolic compounds, especially flavonoids, in legume root exudates, in conjunction with rhizobial NodD proteins, activate the expression of bacterial nodulation genes (*nod*, *nol*, and *noe*) involved in the early stage of nodule formation (13). Most *nod* genes are involved in the synthesis and secretion of a family of lipochitin-oligosaccharides called Nod factors that are required for the rhizobial invasion and initiation of nodule formation on the host roots. To test the functionality of these new vectors, the promoter of the *nodABC* genes of NGR234 was cloned as a 300-bp *Bgl*II-*Sma*I fragment from pNBA (5) into pMIT and pMIG. The ligation product was electroporated into *E. coli* S17-1 λ_{pir}, and the recombinant vectors were transferred by conjugation into NGR234. Transconjugants were selected and purified on RMM plates that contained either gentamicin or tetracycline. Homologous recombination was verified by PCR using the primers pKOuni (5′-TTGCCCTCATCTGTTACGCC-3′) and pKOrev (5′-CCATGTCAGCCGTTAAGTGTTC-3′) for the amplification of an internal fragment that was common to the three vectors. Single colonies were selected and screened by colony PCR that was carried out in a total volume of 20 µL containing 2 µL boiled bacterial colonies in 100 µL sterile double-distilled water, 250 µM dNTPs, 2 µL 10× *Taq* DNA polymerase (Qiagen, Valencia, CA, USA) reaction buffer, 0.5 µM each primer, and 0.5 U *Taq* DNA polymerase. The PCR mixture was held at 97°C for 10 min and then cycled 30 times at 94°C for 30 s, 55°C for 45 s, and 72°C for 25 s, followed by 5 min at 72°C in the final cycle. Positive clones were tested for induction of chloramphenicol-resistance and fluorescence following the addition of the flavonoids. When cultures of the transconjugants growing in RMM media had reached an absorbance (A_{600}) of 0.1, apigenin or daidzein (Sigma, St. Louis, MO, USA) was added to a final concentration of 10^{-6} and 10^{-9} M, respectively. Two hours later, chloramphenicol was added to a final concentration of 75 µg/mL. The fluorescence of the cultures was scored 36 hours later

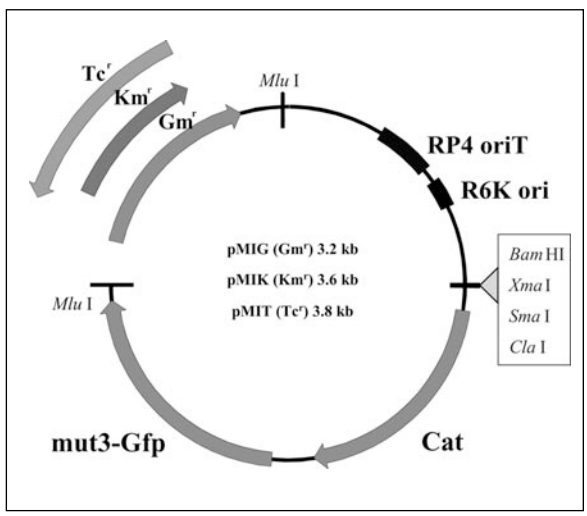

Figure 1. Plasmid map of the pMIG, pMIT, and pMIK vectors. (Vectors are not drawn to scale.) Tc, Gm, and Km confer resistance to tetracycline, gentamicin, and kanamycin, respectively. Arrows indicate the location and orientation of gene expression. The black boxes mark the origin of replication of the R6K plasmid (R6K ori) and the mobilization fragment (RP4 oriT).

using a Aminco Bowman® Series 2 luminescence spectrometer (Spectronic Instruments, Rochester, NY, USA), with an excitation wavelength of 475 nm and an emission wavelength of 512 nm. A random transcriptional fusion library of NGR234 DNA digested with *Mbo*I was also constructed in pMIG. After mobilization into the parent strain NGR234, the colonies were selected on TY medium containing rifampicin and gentamicin and visualized using the Dark Reader™ blue transilluminator (Clare Chemical Research, Dolores, CO, USA).

Table 2. Fluorescence of Flavonoid-Induced and Non-Induced Cultures of *Rhizobium* sp. NGR234 Transconjugants

	Relative Fluorescence[a]		
	Non-induced	Apigenin-induced	Daidzein-induced
NGR234(pMIG::*nodABC*)	0.82 ± 0.02	3.37 ± 0.04	3.64 ± 0.35
NGR234(pMIT::*nodABC*)	0.85 ± 0.1	4.17 ± 0.02	3.22 ± 0.05
NGR234	0.47 ± 0.01	0.65 ± 0.10	0.45 ± 0.03

[a]Relative fluorescence units measured by taking the mean measurement at 20, 40, and 60 s. Values are $\bar{x}$ ± SD. NGR234(pMIG::*nodABC*) and NGR234(pMIT::*nodABC*) correspond to NGR234 exconjugants containing pMIG or pMIT, respectively, harboring the promoter of *nodABC*.

RESULTS

The two vectors tested, pMIG::*nodABC* and pMIT::*nodABC*, gave high frequencies of conjugation (10^{-3} per recipient cell). The addition of apigenin or daidzein induced resistance to chloramphenicol. The growth of the flavonoid- and chloramphenicol-treated cells containing pMIG::*nodABC* or pMIT::*nodABC* was similar to that of a non-induced cultures without the addition of chloramphenicol. However, only the exconjugants showed fluorescence after flavonoid addition. The fluorescence of the non-induced exconjugants was similar to that of wild-type NGR234 (Table 2), suggesting that transcription of *mut3-gfp* is suppressed in the absence of flavonoid activation of the promoter of *nodABC*. The simultaneous antibiotic resistance and fluorescence of the bacterial culture show that both the *cat* and *mut3-gfp* genes are co-expressed. Either one of these genes might be used as a reporter for gene activity, using chloramphenicol resistance or fluorescence as measurable phenotypes. The cointegrated NGR234 library was observed under blue light, and we could easily discriminate directly on the culture plate colonies different fluorescence intensities (Figure 2A) that allowed us to assess a priori the functional status of the transcriptional fusions. The fluorescent colony NGR234::pMIG2517 was randomly selected (Figure 2B), purified, and the transcriptional fusion was recovered from the chromosome by conjugative cloning and sequenced. The transcriptional fusion showed a high similarity to *nuoG*, which encodes for a peripheral subunit of the NADH dehydrogenase I complex that couples the oxidation of NADH to the generation of proton motive force (4). The expression of *nuoG* is involved in the bacterial respiration and is essential for complex I function (16). The fluorescence of NGR::pMIG2517 shows the capacity of the vector pMIG to report fusions that are highly expressed, as observed with *nuoG*.

The co-integrated NGR234 library

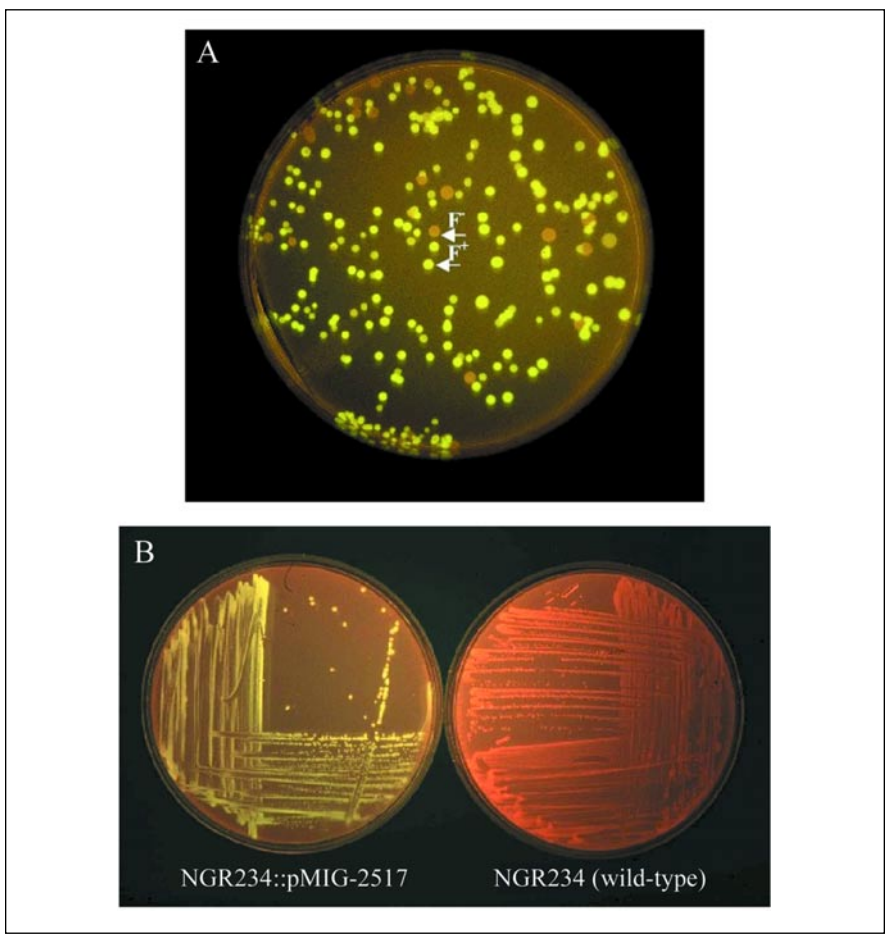

Figure 2. Direct visualization of fluorescence under blue light. (A) The difference in the intensity of the fluorescence is shown for a genomic library of *Rhizobium* sp. NGR234 cloned in pMIG that has been mobilized back into *Rhizobium* sp. NGR234. Strains carrying active promoters cloned upstream of the reporter cartridge appear highly fluorescent (F^+), while inactive promoters or promoterless fusions are unable to stimulate fluorescence (F^-). (B) The clear differences in the fluorescence of a highly fluorescent exconjugant (NGR234::pMIG-2517) that shows high similarity to *nuoG* and the parental strain *Rhizobium* sp. NGR234.

was used to study the colonization process of the plant root system by NGR234, and the details of the fusion strains induced specifically in the rhizosphere will be reported elsewhere.

This set of vectors, pMIK, pMIG, and pMIT are broad-host-range mobilizable plasmids for promoter trapping. They are derivatives of pKNOCK vectors, whose replication require the presence of the π replication protein and can therefore be maintained as an extrachromosomal element only in hosts containing the *pir* gene. pMIK, pMIG, and pMIT also carry the RP4 origin of conjugal transfer that allows their mobilization into other Gram-negative bacteria when the RP4 transfer functions are provided in *trans*. Hence, the new vectors can be propagated in *E. coli* and transferred to a wide range of Gram-negative bacteria by means of either conjugation or electroporation. Integration into the genome of various hosts via homologous recombination can then be forced. Because these vectors contain a promoterless *cat* gene that can be used to select for inducible promoters (11,12), they will be valuable tools in bacteria with no known attenuated auxotrophy. Furthermore, the promoterless *mut3-gfp* gene with enhanced fluorescence allows the monitoring of transcription levels without invasive procedures both in vivo and in vitro (7,19). By integrating these plasmids into the host-chromosome, artifacts associated with multicopy plasmids can be eliminated. The sites of chromosomal integrations can be recovered by conjugative cloning (15), and the *cat-mut3gfp* fusion junction can be sequenced using a primer homologous to the 5′-end of the *cat* gene that reads upstream into the cloned sequence. An additional advantage is that their relatively small size facilitates the construction of representative genomic libraries. This new set of vectors offers the possibility to quantify the transcriptional activity of randomly cloned promoters using either fluorescence measurement or *cat* production. The presence of an improved GFP gene allows the use of pMIG, pMIK, and pMIT in a wide range of environments (2,20). Additionally, they can be used in gene knockout by insertional mutagenesis provided that a functional upstream promoter is cloned in the polylinker.

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