Genetic Tools for Pseudomonads, Rhizobia, and Other Gram-Negative Bacteria

BioTechniques 32:386-401 (February 2002)

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ABSTRACT

*Gram-negative bacteria are extraordinarily diverse microorganisms that present a wide variety of characteristics worthy of genetic investigation. For historical reasons, the application of recombinant DNA technology to Gram-negative bacteria in general has always lagged behind that of *E. coli* and its close relatives. However, the past 10 years have seen dramatic advances in the development of new tools and vectors for genetic analysis in non-*E. coli* hosts. Applications include various kinds of genetic manipulation, conjugation, transposition, site-specific recombination, protein secretion, protein purification, cell suicide, microbial ecology, biodegradation, and plant and animal pathogenicity.*

INTRODUCTION

The recombinant DNA revolution began in *E. coli* in the mid 1970s and evolved rapidly because the well-studied plasmids (ColE1, p15A, and pSC101) and bacteriophage λ needed little modification for use as recombinant DNA vectors. Thus, recombinant DNA technology rapidly produced a wide variety of sophisticated special-purpose vectors for *E. coli*. On the other hand, recombinant DNA opened up the prospect of performing genetic analyses in a wide variety of other bacteria that present myriad features of scientific, medical, veterinary, agricultural, and biotechnological interest. Among such bacteria are species of the genera *Acetobacter*, *Acinetobacter*, *Agrobacterium*, *Alcaligenes*, *Azotobacter*, *Bordetella*, *Bradyrhizobium*, *Brucella*, *Burkholderia*, *Comamonas*, *Methylobacterium*, *Paracoccus*, *Pseudomonas*, *Ralstonia*, *Rhizobium*, *Rhodopseudomonas*, *Sinorhizobium*, *Sphingomonas*, *Vibrio*, *Xanthomonas*, and *Yersinia*. Unfortunately, the narrow-host-range *E. coli* vectors are unable to replicate in most of these bacteria, and alternative cloning systems had to be found. The purpose of the present review is to summarize the state-of-the-art genetic tools for recombinant DNA in Gram-negative bacteria other than *E. coli*.

GENERAL CLONING VECTORS

Genetic engineering with Gram-negative bacteria has a number of require-

ments that vary in importance according to the specific experimental situation. Table 1 provides useful, general-purpose plasmid vectors characterized according to their replicon, cloning characteristics, and selective markers.

Replicon

In general, all vectors need to replicate in the chosen host (major exceptions are transposon vectors and certain promoter-probe vectors, which integrate into the host chromosome). Using broad-host-range replicons as the basis of cloning vector development (29,31, 106) has the advantage that the cloning may be performed by standard techniques in *E. coli*, which is easy to manipulate, and the recombinant plasmid is subsequently transferred to different experimental hosts, usually by conjugation. Historically, three main types of broad-host-range replicons have been popular for vector construction: RK2 (IncP), RSF1010 (IncQ), and pSa (IncW). These plasmids are able to replicate in a wide variety of Gram-negative hosts. Indeed, the RSF1010-based, cosmid cloning vector pJRD215 (27,31), though originally constructed for *Pseudomonas* spp., has also proven useful in most Gram-negative bacteria and some Gram-positives, including *Pasteurella multocida*, *Porphyromonas gingivalis*, *Actinomyces naeslundii*, *A. visosus*, *Thiobacillus ferrooxidans*, *Mycobacterium aurum*, *M. smegmatis*, *Rhodobacter sphaeroides*, *Desulfovibrio vulgaris*, *Streptomyces lividans*, *Brevibacterium methylcum*, and the marine

Table 1. General-Purpose Cloning Vectors

Cloning Vector	Replicon	Characteristics	Reference
pRK310 series	RK2	Tc ^R , <i>lacZ</i> α, <i>mob</i> , IncP	(43)
pLAFR5	RK2	Tc ^R , <i>mob</i> , <i>mcs</i> , <i>cos</i> , IncP	(70)
pRK415	RK2	Km ^R , <i>lacZ</i> α, <i>mob</i> , <i>mcs</i> , IncP	(70)
pJB3Cm6 series	RK2	Cm ^R , Ap ^R , <i>lacZ</i> α, <i>mcs</i> , <i>oriT</i> , IncP	(15)
pKT210 series	RSF1010	Cm ^R , Sm ^R , IncQ	(10)
pJRD215	RSF1010	Km ^R , Sm ^R , <i>mcs</i> , <i>mob</i> , <i>cos</i> , IncQ	(31)
pDSK509 series	RSF1010	Km ^R , <i>mcs</i> , <i>mob</i> , IncQ	(70)
pUFR027 series	pSa	Km ^R , <i>lacZ</i> α, <i>mcs</i> , <i>mob</i> , IncW	(37)
pBBR1MCS-1 series	pBBR1	Cm ^R , <i>lacZ</i> α, <i>mcs</i> , <i>mob</i>	(12, 41, 75, 76)
pUCP18 series	pMB1/pR01614	Ap ^R , <i>lacZ</i> α, <i>mcs</i>	(110, 111)
pVSP41 series	p15A/pVS1	Km ^R , <i>sta</i> , <i>cos</i> , <i>mcs</i>	(130)
pME6010 series	p15A/pVS1	Tc ^R , <i>sta</i> , <i>mcs</i> , <i>oriT</i>	(54)
pRAD1	pUE10/pMLT23	Ap ^R , Cm ^R , <i>lacZ</i> α, <i>mcs</i>	(86)

No attempt is made to list all available cloning vectors. When a series of similar variants is available this is indicated by "series" in column 1. Vectors are grouped according to their replicon. Abbreviations are : Tc^R, resistance to tetracycline; Km^R, resistance to kanamycin; Cm^R, resistance to chloramphenicol; Ap^R, resistance to ampicillin; Gm^R, resistance to gentamycin; Tp^R, resistance to trimethoprim; Hy^R, resistance to hygromycin; Sp^R, resistance to spectinomycin; *sta*, stability locus; *cos*, cosmid (cohesive ends of phage λ); *mcs*, multiple cloning site; *lacZ*α, β-galactosidase complementation; *parDE* plasmid maintenance; *oriT*, origin of transfer; *mob*, mobilization; functions of RSF1010; *inc*, incompatibility group.

cyanobacterium *Synechococcus* sp.

The RSF1010- and RK2 (RP4)-based cloning vectors are typically larger in size than the narrow-host-range, pMB1-based cloning vectors of *E. coli*. The size of the RSF1010-based vectors cannot easily be reduced since three genes (*rep*) plus the origin of replication (*oriV*) are necessary for the replication, and three *mob* genes plus the origin of transfer (*oriT*) are needed for the mobilization (Tables 1–3). In RK2-based vectors, the minimal replicon for replication contains only the *oriV* and the *trfA* genes, but in addition to *oriT* for conjugal transfer (i.e., when the Tra functions are provided in trans), it is also preferable to include one or more plasmid stabilization functions such as *parDE*. A more complete understanding of the molecular biology of plasmid RK2 has now permitted the construction of smaller well-defined RK2 vectors (15,16).

More recently, several alternative replicons have also been used for vector construction. A newly discovered broad-host-range plasmid, pBBR1 (from *B. bronchioseptica*), has the ad-

vantage of having a small and stable minimal replicon (12,41,75,76). Similarly, the *Pseudomonas* plasmid pVS1 is able to replicate in many Gram-negative bacteria but not in *E. coli* (130). The minimal replicon of pVS1 was combined with *E. coli* p15A replicon, thus adding the considerable advantages offered by this host. The resulting plasmids are relatively small in size and are quite stable, even under nonselective conditions (54). Yet another series of vectors is based on replicon pRO1600, which, like VS1, can replicate in many Gram-negative species but not in *E. coli*. This plasmid was originally thought to be derived from RP1, but recent sequencing of the minimal origin of replication now makes this unlikely. The combination of the minimal origin of pRO1600 with the *E. coli* pUC18/19 produces vectors able to function in *E. coli* and in *Pseudomonas* (110,135).

Deinococcus radiodurans is not related to any bacterium whose genetics are well understood but has proven to be particularly refractile to gene cloning, probably because its promot-

ers are not recognized in *E. coli* and visa versa. Double replicon, *D. radiodurans*-*E. coli* shuttle vectors have been constructed by making hybrids between pBR322-based plasmids and a naturally occurring *D. radiodurans* plasmid pUE10 (84,86).

Incompatibility

Plasmids are of the same incompatibility group when they cannot be stably maintained in the same host. Conversely, plasmids from different incompatibility groups (Inc), such as incQ, incP, and incW (Table 1), are compatible and may stably co-replicate in the same cell. This can be important when it is desired to supply genetic functions *in trans* from different plasmids (27,28). It is also useful for the elimination of a resident plasmid, for example, during gene replacement (126).

Conjugation

Many Gram-negative bacteria are difficult to transform with naked DNA, whereas conjugation is usually quite ef-

ficient. In most cases, broad-host-range vectors contain an origin of conjugal transfer, usually the *oriT* of plasmid RK2 or the *mob* region of RSF1010 (26,27,106). To facilitate vector construction, special cassettes containing the *oriT* region of plasmid RP4 have been constructed (4). Mobilizable vectors are small and incapable of self-transfer but can be mobilized in the presence of a conjugative plasmid such as RP4. For this purpose, the *E. coli* strains S17-1 or SM10 (116), which have a chromosomally integrated copy of RP4 able to supply the transfer functions *in trans*, may be used. In other hosts, a tri-parental mating system using plasmid pRK2013, a ColE1-based plasmid carrying the *tra* genes of RK2, is employed (106).

Selective Markers

The cloning vectors all contain antibiotic resistance markers to select plasmid-containing colonies, following transformation or conjugation. Since different bacteria are sensitive to different antibiotics, a given series of plasmid vectors (Table 1) may have a selection of selective markers appropriate to a given host. Numerous cassettes containing antibiotic resistance genes flanked by unique restriction sites have been constructed (3,5,41,50).

Cosmids

Many broad-host-range cloning vectors contain *cos* sites, which are short regions of DNA containing the cohesive ends of phage λ . These vectors are referred to as cosmids and have the advantage that they can accept large fragments of DNA. Thus, 30–48-kb fragments (depending on the size of the cosmid vector) from a partial digest of chromosomal DNA are ligated to the cosmid vector and efficiently packaged into bacteriophage λ particles. These phage particles can then be used to inject the packaged DNA into *E. coli*, where it replicates as a plasmid. When the *E. coli* host (e.g., S17-1) carries a RK2 plasmid, the resulting plasmids may then be transferred to another bacterium (e.g., *Pseudomonas*) by conjugation. This permits the rapid screening of an entire gene bank and selection of

Table 2. Expression Vectors

Expression Vector	Replicon	Characteristics	Reference
pVDZ'2	RK2	<i>Plac</i> , Tc ^R	(40)
pJB864 series	RK2	<i>xyIS Pm</i> , Ap ^R , <i>mcs</i>	(15, 16)
pCF430	RK2	<i>araC P_{BAD} Tc^R, mcs, mob</i>	(94)
pMMB22 series	RSF1010	<i>lacI^q, Ptac</i> , Sm ^R , Ap ^R	(11, 92)
pVDtac39 series	RSF1010	<i>lacI^q, Ptac</i> , Ap ^R , <i>mcs</i>	(40)
pNM185	RSF1010	<i>xyIS Pm</i> , Km ^R , Sm ^R , <i>mcs</i>	(87)
pML122	RSF1010	<i>PNm</i> , Km ^R , Tc ^R	(78)
pML130	RFS1010	<i>Plac</i> , Km ^R , Tc ^R	(78)
pJRD215K	RSF1010	λ cl ₈₅₇ , λ P _R , Km ^R , Sm ^R , <i>mob</i>	(30)
pPLGN1	RSF1010	λ cl ₈₅₇ , λ P _L , Km ^R , Sm ^R , <i>mob</i>	(80)
pJRD253	RFS1010	phage T7 ϕ 10, Sm ^R , <i>mob</i>	(28)
pUCPKS	pMB1/pR01614	phage T7 ϕ 10, Ap ^R , <i>lacZα</i> , <i>mcs</i>	(133)
pJN105	pBBR1	<i>araC P_{BAD} Gm^R, mcs, mob</i>	(94)
pBBad22T series	pBBR1	<i>araC P_{BAD} Tc^R, mcs, mob</i>	(121)
pBBad22K	pBBR1	<i>araC P_{BAD} Km^R, mcs, mob</i>	(121)
pSPORTn3	pMB8	Tn3-based Ap ^r , <i>lacP</i> , P _{lac}	(69)

No attempt is made to list all expression plasmids. When a series of similar variants is available this is indicated by "series" in column 1. Vectors are grouped according to incompatibility group. Transposon expression vectors are listed under Table 3. Abbreviations are as in Table 1 except: *lacI^q* and *Ptac* and *Plac*, IPTG-responsive regulator and promoters; *xyIS* and *Pm*, alkylbenzoate responsive regulator and promoter; *araC* and *P_{BAD}* arabinose responsive regulator and promoter; λ cl₈₅₇ and λ P_R and λ P_L; thermosensitive λ repressor and promoters; phage T7 ϕ 10, T7 polymerase responsive promoter.

a recombinant clone by complementation of a mutant host (19,27,29,31,106).

Multiple Cloning Sites

It is usually desirable for a cloning vector to contain unique restriction sites for a variety of different restriction enzymes (31). Often, in *E. coli* vectors, these restriction sites are clustered and associated with the *lac* gene fragment that permits α -complementation for β -galactosidase activity in a suitable (Δ M15 *lacZ* deletion) *E. coli* host. This results in the well-known blue/white colony discrimination on X-gal IPTG plates. More recently, *Pseudomonas* strains, containing an *E. coli* DNA fragment carrying the Δ M15 *lacZ* deletion, have been constructed and permit visualization of the blue/white colony phenotype with vectors capable of α -complementation (69).

EXPRESSION VECTORS

A number of different systems have been constructed to facilitate the artificial regulation and over-expression of a cloned gene (Table 2). The classical systems are based upon tried and tested regulator/promoter systems from *E. coli*, such as the *P_{lac}* or *P_{tac}* promoters and LacI repressor, inducible by IPTG promoters (11,40); the thermo-sensitive lambda λ cl₈₅₇ repressor, with promoters λ p_R and λ p_L (30,80); and the arabinose repressor, AraC, with the promoter *P_{BAD}* induced by arabinose (94, 121). Recent improvements in RK2 vectors have led to new expression vectors based on the *Pu/XylR* regulator (inducible by xylenes and toluene) and on the *Pm/XylS* regulator (inducible by alkylbenzoates) (15,16). The coliphage T7 ϕ 10 promoter is also extremely efficient in a wide variety of bacteria when

the T7 RNA polymerase is supplied *in trans* from a compatible plasmid (27,28) or from a transposon integrated into the host chromosome (32,34,58, 133). Another advantage of the T7 promoter/polymerase system is that the proteins produced under its control continue to be synthesized in the presence of rifampicin, an inhibitor of the host RNA polymerase. Thus, recombinant proteins can be specifically labeled and visualized by SDS-PAGE (28).

A further development of the T7 promoter system placed the T7 polymerase gene under control of the *P_m* promoter, which is positively induced by XylS protein in the presence of alkylbenzoates. Both the *xylS* gene and the *P_m-T7pol* fusion were carried by a mini-Tn5 transposon that could be inserted into the chromosome of *Pseudomonas putida*. Target genes for the T7 polymerase were placed under the control of the T7 promoter and carried by multiple mini-transposons elsewhere on the *P. putida* chromosome. Thus, induction by alkyl-benzoate could provoke the simultaneous production of different target proteins in response to a single-inducer molecule in the environment (58). The *xylS* system permits excellent control of gene expression in many different bacteria and may have advantages over the *lac* and λ systems, which are difficult to manipulate for environmental and large-scale purposes.

A useful tool for the purification of proteins overproduced in recombinant bacteria utilizes the 6×his-tagging system that permits proteins to be purified by metal affinity columns (14).

TRANSPOSON TOOLS

Transposons are mobile broad-host-range genetic elements that can be transferred between bacteria by conjugation, transduction, and transformation. With the advent of genetic engineering, transposons have often been modified to construct special-purpose genetic tools (Table 3). The seminal work of Simon and co-workers (116) showed that a specially designed transposon delivery system could offer enormous advantages for transposon-mediated insertional mutagenesis. They constructed derivatives of narrow-host-

Table 3. Transposon Vectors

Transposon	Properties	Reference
Tn5-TC1 (series)	Tc ^R	(109)
Tn5-B10 (series)	Nm ^R , <i>mob</i>	(116)
Tn5-B60	Nm ^R , <i>P_{tac}</i> mobile promoter	(116)
Tn5-K28 (series)	Tc ^R , Sm ^R , mcs	(67)
Tn5-30Tp	Km ^R , Tp ^R	(1)
Tn5-Hy (series)	Hy ^R	(12)
mini-Tn5-Cm (large series)	Cm ^R , <i>tnp⁻</i>	(34, 35, 59)
mini-Tn10-Km (large series)	Km ^R , <i>tnp⁻</i>	(34, 35, 59)
mini-Tn5xylS/ <i>P_m::T7pol</i>	Km ^R , <i>tnp⁻</i> , <i>xylS/P_m</i> promoter, T7 RNA polymerase	(58)
Tn1722-299Km	Km ^R , Tc ^R , Cm ^R , <i>ori_{pACYC184}</i>	(127)
pTnMod-OCm (large series)	Cm ^R , <i>tnp⁻</i> , <i>ori_{pMB9}</i>	(39)
pTn5 <i>cat</i>	Nm ^R , promoterless <i>cat^R</i> , <i>mob</i> , <i>tnp⁻</i> , <i>ori_{ColE1}</i>	(83)
Tn5-OT182	Tc ^R , Ap ^R , promoterless <i>lacZ</i> , <i>ori_{ColE1}</i>	(88)
mini- <i>OphoA</i>	Gm ^R , <i>phoA</i> , <i>ori_{pMB1}</i>	(18, 123)
No attempt is made to list all transposons. When a series of similar variants is available this is indicated by "series" in column 1. Abbreviations are as in Tables 1 and 2 except for <i>tnp⁻</i> , transposase negative transposon; <i>ori_{pACYC184}</i> , origin of replication of plasmid pACYC184; <i>ori_{pMB9}</i> , origin of replication of plasmid pMB9; <i>ori_{ColE1}</i> , origin of replication of plasmid ColE1.		

range *E. coli*-specific plasmids pACYC184 and pBR325 that contained the *mob* region of RK2 and could thus be transferred, by conjugation, to many different Gram-negative bacteria. The plasmids could not replicate in these foreign bacteria and thus acted as suicide plasmids. However, when the *mob* plasmids were also equipped with a transposon such as Tn5 or Tn10, the transposon could survive, at low frequency, by transposition to the host chromosome. An alternative transposon delivery technique relies on the narrow-host-range replicon of plasmid R6K, which can replicate when the replication protein π is provided *in trans* but acts as a suicide plasmid when transferred to a strain lacking this protein (89). Irrespective of the delivery vehicle, the *E. coli* strain S17-1, containing an integrated conjugation proficient RP4 plasmid (116), is usually used. To permit the replication of the

suicide R6K replicon, S17-1 is made lysogenic for λ pir, which encodes the replication protein π (73,89).

The obvious utility of transposons prompted the construction of modified variants of Tn5, containing different combinations of antibiotic resistance genes (1,12,109). Variants containing the RP4 *mob* region were also constructed (116). The Tn5 transposon was modified for use as a cloning vector by maximizing the number of restriction sites inside the Tn5 transposon and eliminating those within the carrier plasmid. Thus, foreign DNA could be cloned more easily into the transposon and was subsequently transposed, along with the transposon, into the chromosome of the recipient bacteria, following conjugation (27,67). Bacteria carrying foreign DNA cloned within a transposon have the advantage of being stable in the absence of continued antibiotic selection. This is necessary for

many types of environmental applications (e.g., bioremediation or biocontrol, by the species of *Pseudomonas*, *Sphingomonas*, *Ralstonia*, *Comamonas*, and *Burkholderia* or nitrogen fixation, by the species of *Rhizobium*, *Azorhizobium*, and *Bradyrhizobium*).

A large series of mini-transposon vectors was designed in which Tn5 transposons were reduced to a minimum size; they essentially consisted of only the two IS50 terminal sequences and a selective marker (Table 3). Another useful characteristic of this mini-transposon series is the presence in each transposon of unique sites for rare-cutting restriction enzymes, *NotI* and *SfiI*, into which foreign DNA may be cloned (35,36,59). The transposase gene was located on the suicide plasmid adjacent to, but outside of, the mini-transposon so that transposition results in the dissociation of the transposon from its transposase gene. This further enhances genetic stability, prevents secondary transposition, and thus limits horizontal transfer. (35,36,59).

A unique kind of transposon was based on the narrow-host-range *E. coli* pMB8 replicon. By the insertion of a second 38-bp Tn3 inverted repeat, pMB8-based vectors were converted into transposons that could form co-integrates with any broad-host-range plasmid and then be transferred to alternate hosts by conjugation (72).

Various modified transposons contain an origin of replication that is functional in certain hosts but not others. Thus, they are plasmids in the permissive host but can exist only as integrated transposons in the non-permissive host. These are generally referred to as self-cloning vectors or plasposons because they form plasmids when they are excised from the non-permissive host, are re-ligated, and transformed into the permissive host. One series of modular mini-transposons (Table 3, pTnMod) is based on the RK6 origin of replication (39). Thus, an RK6-based pTnmod plasposon inserted into, for example, the *B. cepacia* genome may be excised with an appropriate restriction enzyme, circularized using DNA ligase, and then transformed into the permissive host *E. coli* (λ pir). Using these self-cloning vectors, the gene into which the plasposon is inserted may be cloned and sequenced in

as few as two days. Since plasposons carry sites for rare-cutting restriction enzymes, they also have potential applications for the physical mapping of unknown genomes and the preparation of large restriction fragments.

A similar Tn5-based plasmid/transposon, pTn5cat, carrying the origin of replication of ColE1, the *mob* region of RP4, and sites for the rare-cutting restriction enzymes (83,88) has also been described. Another self-cloning vector is based on a transposon Tn1722, variant Tn1722-299Km, which carries the *E. coli*-specific plasmid pACYC184 (1). More recently, a self-cloning system coupled to a *phoA* promoter-probe vector (123) to facilitate cloning of secreted proteins was constructed (18).

With the increased availability of completely sequenced bacterial genomes, new methods are necessary for the mass identification of essential genes. A new technique (136) exploits the highly efficient in vivo transposition of the newly developed mariner transposon. With this system, one transposition event can be obtained per 200 recipients in *P. aeruginosa*, and the genetic locations of the insertion sites can be mass-screened by gel electrophoresis, following PCR. The absence of a transposon insertion in a particular gene (defined by the genomic sequence) provides good evidence that the gene is essential for growth under the conditions selected. This presumptive result is then confirmed by an efficient site-specific recombination method of gene replacement (see section on gene replacement).

INDICATOR GENES AND PROMOTER-PROBE VECTORS

For a great many years, indicator genes have proved invaluable for the genetics of *E. coli*. There are two principal reasons for using indicator genes. First, promoterless indicator genes are useful for studying the control of gene activity (the promoter-probe vectors discussed in this section). Second, indicator genes serve to genetically mark a particular bacterium so that it can be uniquely identified, selected, and quantified at a later time.

Transcriptional fusions of indicator genes provide information on the genet-

ic control of the gene to which they are fused. Similarly, translational fusions provide information on posttranslational processing, protein secretion, and localization in the cell. In many cases, a promoter-probe plasmid contains multiple cloning sites upstream of a promoterless indicator gene so that cloning of specific or random fragments carrying promoter activity will result in gene expression under appropriate conditions in the relevant host (105,106). In other cases, homologous recombination with the chromosomal gene, rather than cloning, is used to accomplish the fusion event (65). In still other cases, the promoter-probe plasmid may be a mini-transposon that randomly inserts into the host chromosome and frequently causes fusions with an active promoter (34). The latter two systems have the advantage that the gene fusion is similar to a single copy in its normal chromosomal location, thereby avoiding potential problems caused by position or plasmid copy number. Transposon Tn5 has been the most popular transposon used in promoter-probe constructions, but others include Tn3 (85). The transposon IS903 ϕ kan (124) is also worthy of special mention because not only is its transposase located outside of the transposon but also it is under the control of the *lac* promoter. This means that transposition can be induced by IPTG and detected by the resistance to kanamycin. IS903 ϕ kan is therefore a useful tool for bacteria that are poor recipients for transformation or conjugation.

The classical reporter gene that was first used in *E. coli* genetics is *lacZ*, which codes for β -galactosidase. Promoter-probe vectors carrying *lacZ* have been successfully applied to *Pseudomonas*, *Rhizobium* and other Gram-negative bacteria (33,64,65,71,78,88, 116). The much smaller *lacZ* α fragment has also been used (105), although this system requires specially designed bacteria that carry a DNA fragment of *E. coli*, which contains the complementing Δ M15 *lacZ* deletion (69). Many other promoterless indicator genes have been used in promoter-probe plasmid vectors, including antibiotic resistance genes for streptomycin (11), tetracycline (48), chloramphenicol (47,83,96), kanamycin (124), gentamycin (116), and ampicillin (64).

Other promoterless indicator genes include: *xylE*, catechol 2,3-dioxygenase from *P. putida* (64,67,74); *galK*, galactokinase from *E. coli* (30); *inaZ*, ice nucleation activity (81); *luxAB* or *luxE-CABD* luciferase from *Vibrio fischeri* (35,48,66,134); *luc*, luciferase from fireflies (97); *gfp* from *Aequorea victoria* (52,85,96,120); *gusA*, β -glucuronidase of *E. coli* (103); *celB*, thermostable β -glucosidase of *Pyrococcus furiosus* (114); and *phoA*, alkaline phosphatase from *E. coli* (18,48,64,123). Each of these systems offers some advantages, such as selection, ease of enzyme quantification, color reaction, light emission or fluorescence. The alkaline phosphatase protein-fusion transposon has the advantage that the enzyme is active only after export from the cytoplasm, which allows for the enrichment of insertions into genes that encode secreted proteins (18,123). Recently, GFP has replaced many other indicator systems because of the many advantages it offers.

IN VIVO EXPRESSION TECHNOLOGY

There is enormous interest in knowing which genes are specifically expressed by bacterial pathogens during pathogenesis. Building on the observation that these genes are quite different from those expressed by the same pathogen during growth in the laboratory, two new and related methods, in vivo expression technology (IVET) and recombinase-based in vivo expression technology (RIVET), have been devised to identify genes that are expressed in the host during pathogenesis. These important innovations have been the subject of extensive reviews (20,21,56,82,118) and will be dealt with only briefly here. Both are based on promoter-probe plasmids that permit the identification of promoters that are active during pathogenesis. In IVET and RIVET vectors, potential promoter-containing DNA fragments from pathogenic microorganisms are cloned in front of a promoterless reporter gene and its in vivo expression profile is then monitored.

In the case of IVET, the reporter gene is essential for pathogenic growth

in the host. Such genes include metabolic genes, such as *purA* or *thyA*, respectively, which are necessary for purine and thymine biosynthesis (118,119), and *asd*, which is necessary for diaminopimelate biosynthesis (52). The recipient host carries a deletion for the corresponding gene to permit genetic complementation. Antibiotic resistance genes, such as *cat*, chloramphenicol resistance (82), and *tmp*, trimethoprim resistance (115), where the host is treated with the appropriate antibiotic, have also been used.

In addition to these selective genes, the expression of visible indicator genes may be simultaneously monitored (52). GFP may be particularly interesting for noninvasive real-time monitoring. A technique known as differential fluorescence induction uses a promoter-trap strategy involving GFP coupled with semiautomated fluorescence-activated cell sorting to directly isolate in vivo pathogens that express the indicator gene (128,129).

For both IVET and RIVET, the promoterless reporter genes carried by an *ori*-RK6 suicide plasmid are fused to the pool of promoter fragments from the pathogenic bacterium in vitro before transformation of *E. coli*-(λ pir). It is necessary to use a fusion library large enough so that there would be a 99% probability of fusion for every gene in the bacterium. The transformation pool is then conjugated to the recipient pathogen. In the pathogen, the transferred *ori*-R6K plasmid does not replicate, and integration must thus take place by Campbell-type homologous recombination between the promoter-containing fragment and the corresponding region of the host chromosome. This Campbell-type insertion is additive so that the host gene is neither inactivated nor deregulated in the process.

The RIVET technology uses a different kind of promoterless reporter gene compared to IVET. Instead of a gene essential to the host survival, RIVET uses a promoterless site-specific recombinase gene [e.g., the *E. coli* Tn γ δ resolvase (21) or the *cre* recombinase of phage P1 (6), whose expression is of no consequence to the survival of the pathogen]. The expression of the site-specific recombinase is monitored by its ability to excise indicator genes

(e.g., Km^R, kanamycin resistance, and *sacB*, sucrose sensitivity) located between site-specific recombination sites (*res* sites for $\gamma\delta$ resolvase and *lox* sites for *cre* recombinase) artificially inserted elsewhere on the bacterial chromosome. This has the advantage that RIVET can be used to identify genes that are temporally expressed during pathogenesis or that are tissue-specific.

In both IVET and RIVET, the authenticity of the putative pathogen genes expressed in vivo are subsequently verified by more classical techniques such as DNA sequencing, amino acid sequence comparisons, and mutation and deletion analysis.

ENVIRONMENTAL MARKER GENES

Recent years have seen an increased interest in the role of microorganisms in the environment. To experimentally investigate microbial interactions in a natural ecosystem, it is often necessary to introduce specific bacteria into the environment or into an artificial microcosm. For this purpose, the bacteria must be genetically marked so that they can be detected and quantified during the experiment (62,99). Often, this genetic marking is performed by the insertion of mini-transposons (33), although, occasionally, stable plasmids are used (12). Numerous strategies have been devised to genetically mark these transposons. Usually, these include a combination of selectable markers to facilitate the recovery of the bacteria on Petri dishes. The selectable marker is often an antibiotic resistance gene (12), but for environmentally friendly reasons, other selective markers have been used (e.g., resistance to the herbicide Bialaphos, to mercuric salts and organomercuric compounds, and to arsenite) (59). Some of these non-antibiotic markers are not entirely satisfactory because of problems of spontaneous mutation or high tolerance (108). More recently, new mini-transposons and plasmids have been constructed that use tellurite resistance (108) and nickel resistance (122). Both of these systems give excellent selection with low background. In *Pseudomonas*, bacteria carrying specific

siderophore receptors can be specifically isolated from environmental samples using iron-deprived medium supplemented with the appropriate siderophore (100). Still other selective systems may be based on catabolic genes. For *Pseudomonads*, which lack the ability to use lactose as a carbon source, the entire *lac* operon, carried by a plasmid (72) or a mini-transposon (53), has been used as a selective marker. Similarly, in certain *Pseudomonads*, the upper TOL operon, carried by a transposon or a plasmid, may be used as a selective marker. Toluene is converted to benzoate that can then be metabolized to Krebs cycle intermediates by the chromosomal ortho-cleavage pathway (98,108).

Genetic marking usually includes genes for visual screening by an enzymatic color reaction, visible light production, or fluorescence. The most popular visual marker genes are *lacZ*, β -galactosidase (44); *gusA*, β -glucosidase (114); *lux*, bacterial luciferase (131); *luc*, insect luciferase (22); *xylE*, catechol 2-3 dioxygenase (107); GFP (45,85); and *ice*, ice nucleation protein (81). Among these, GFP (45,46) is the most recent and the most popular, since it has numerous advantages over other systems. It is easily and non-destructively detected by illumination with blue or near-UV light and, unlike all other systems, requires no substrate. Individual colonies can be detected with a hand-held, long-wave UV lamp. Initially, the detection of single bacterial cells expressing GFP using epifluorescent microscopy was difficult, but several groups (38,55) have isolated mutants of the *gfp* gene with up to 40 $\times$ enhanced fluorescence. Red-, blue-, and yellow-green-shifted mutants are available and, with suitable filters, can be detected simultaneously in the same sample. GFP has the advantage of being stable to temperature, pH, and denaturing agents. It can thus be detected in dead cells—even those fixed with paraformaldehyde for microscopic observation. For some types of experiments involving real-time changes, this extreme stability can be a disadvantage. To solve this problem, unstable variants of GFP have been constructed that have reduced half-lives compared with the wild-type protein (7). The multitude of

possible applications of GFP (26,45) cannot be summarized here but includes the visualization of the process of infection-thread formation in *R. meliloti* (51), detection of *Pseudomonads* on plant roots (17), monitoring the survival of bioremediation bacteria in polluted soils (46), and conjugation on leaf surfaces (95), in biofilms (23), and in marine bacterial communities (25).

The luciferase genes (*lux* of *V. fischeri* and *luc* of firefly and click beetle) have also been popular for environmental monitoring (99). Both types of luciferase need a substrate, luciferin (a heterocyclic carboxylic acid) for *luc* and long-chain aldehyde for *luxAB*. The remaining *lux* genes (*luxCDE*) are concerned with substrate synthesis and, when present, permit light production in the absence of an exogenously supplied aldehyde substrate. Under favorable conditions, bioluminescent bacterial colonies can be detected by eye in a dark room. More sensitive methods include X-ray films and photography, while the most sensitive methods involve the use of a scintillation counter, a luminometer, or charge-coupled-device-enhanced microscopy. No attempt can be made here to enumerate all of the applications of *lux*-marked bacteria, but some examples are illustrative. Light emission by single bacterial cells was monitored on plant leaves (132) or roots (102). Recombinant *Pseudomonads* carrying the *luxCDABE* genes to the *nahG* promoter were able to produce light when the pollutant naphthalene was present in the medium. This system used fiber optics for the online monitoring of the presence of naphthalene (57,104). Similar systems for the detection of biphenyl and benzene, toluene, ethylbenzene, and xylene were also constructed by fusion to the biphenyl promoter of *R. eutropha* (79) or to the *tod* promoter of *P. putida* F1, respectively (8). *Lux* fusions able to detect the presence of a variety of heavy metals (Ni²⁺, Cu²⁺, Hg²⁺, Cr^{6+/3+}, and Pb²⁺) have also been constructed (24,125,131).

CONDITIONAL LETHALITY AND SUICIDE SYSTEMS

There are a number of systems that

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permit the conditional killing of host bacteria by means of the controlled expression of lethal genes. These systems have two main applications. First, they may be used in the laboratory to eliminate particular bacteria, genetic regions, or plasmids during various forms of gene replacement. Second, they serve to eliminate bacteria in particular environmental situations. Thus, in the case of a bacterium designed for de-pollution, it may be desirable that the bacterium survives only as long as the environment is contaminated by that particular pollutant. Following the complete degradation of the pollutant, the bacterium is programmed to self-destruct. An alternative scenario aims at preventing horizontal transfer to other environmental bacteria. Correctly designed suicide systems may accomplish both of these purposes (90). One such system depends on the *gef* gene of *E. coli*, which,

when it is expressed, kills many different types of bacteria by generating pores in the cell membrane, thereby destroying the membrane potential. To carefully control *gef* expression, a genetic relay was constructed and inserted into a mini-transposon vector. In this system, the *lacI* repressor gene was fused to the *Pm* promoter of *P. putida*, which is controlled by the positive regulator *xylS* that is responsive to 3-methyl-benzoate. Thus, the LacI repressor was synthesized in the presence of 3-methyl-benzoate but not in its absence. The same transposon carried the *gef* gene fused to the *lac* promoter. Thus, the deprivation of 3-methyl-benzoate resulted in derepression of the *lac* promoter and the synthesis of the lethal *gef* gene product. The system provides a model for the suicide of a pollution bioremediation bacterium, following the elimination of the pollutant, and

was shown to work well in the laboratory and in outdoor soil tests (63,91).

A different conditional lethality system was based on the colicin E3 gene (*colE3*), which, when it is expressed, is able to kill a variety of hosts by cleavage of the 16S ribosomal RNA. In a natural situation, this lethality function is counteracted by an immunity function (*immE3*). To examine whether the killing function could be used to prevent the horizontal transfer of plasmid DNA to other bacteria, the *colE3* gene was placed on a plasmid and the *immE3* was placed on the chromosome of *E. coli* or *P. putida*. Thus, in the case of the horizontal transfer of the plasmid to another bacterium, the *colE3* gene would be transferred in the absence of the *immE3* gene, resulting in cell death. This system was able to prevent horizontal transfer to a wide variety of Gram-negative bacteria (42,93,101).

GENE REPLACEMENT, DELETION, AND INSERTION

The complete sequencing of several Gram-negative genomes has revealed large numbers of *fun* (function unknown) genes. The function of these genes might be revealed by their deletion from the chromosome or their replacement by indicator genes to investigate the resulting phenotypic changes. The techniques for allele replacement and gene deletion/knockout (126) all involve the transfer of the cloned gene [(modified by mutation, deletion, or insertion of antibiotic resistance genes, often in the form of Ω -interposons (2,3,49)] to the recipient bacterium on a non-replicating and/or suicide plasmid. The circular plasmid then integrates by Campbell-type homologous recombination. Excision from this merodiploid can occur by spontaneous reversal of the recombination event and, in a proportion of cases, leads to the replacement of the wild-type allele by the mutant allele (126). In some cases, the excision event can be selected by the use of a suicide function [e.g., the *sacB* gene, conferring sensitivity to sucrose (68,112,113) or the *rpsL* gene, conferring dominant streptomycin sensitivity (117)] in the original vector.

A number of different systems have used site-specific recombination to cause in vivo deletion of specific regions of the bacterial chromosome or complex plasmids. Such systems may be useful for precise genetic analysis. They are also particularly useful for the removal of antibiotic genes and other DNA sequences involved in recombinant DNA constructions so that the final strain differs from the parent only in containing the desired genes of interest. A specific deletion in the *trfA* gene of plasmid RK2 was generated by introducing phage P1 *loxP* sites on either side of the gene. The transfer to a strain expressing the P1 *cre* recombinase resulted in the specific deletion of the target gene (9). The multimer resolution system for plasmid partition (*mrs*) on plasmid RP4 was used to generate site-specific deletions in transposons on the *Pseudomonas* chromosome (77). The *mrs* site-specific recombinase acts at two *res* (resolution) sites to excise DNA located between them. In a simi-

lar situation, the *mrs* was used to specifically delete the antibiotic resistance genes from a transposon, following transposition to the chromosome. The result was a quasi-natural strain that differed from the original parent only in having the upper operon of the TOL plasmid pWWO (98).

A system of site-specific plasmid integration of a non-replicating plasmid mini-CTX was devised using the ϕ CTX phage integrase in combination with the appropriate attachment sites. The *Flp*/FRT site-specific recombination of yeast was then used to remove undesired plasmid DNA sequences (13,60,61). Under optimized conditions, these site-specific recombination techniques could be used to create recombinant bacteria that contain only the heterologous gene of interest and are free of plasmid sequences. Such bacteria are desirable for several purposes, including environmental release and live oral vaccines.

Recently, a highly efficient system of site-specific recombination-aided gene replacement was reported by Wong et al. (136). The recipient host bacterium, *P. aeruginosa*, was manipulated to express the *I-SceI* endonuclease, while the conjugally transferred suicide plasmid contained the replacement gene between two *I-SceI* cleavage sites. The presence of the *I-SceI* endonuclease is thought to cleave the incoming plasmid, thus preventing co-integrate formation and possibly stimulating homologous recombination. The method was highly efficient, giving 100% gene replacement.

CONCLUSION

Over the past 10 years, the level of sophistication of recombinant DNA techniques applicable to Gram-negative bacteria has increased to rival those of *E. coli*. The availability of completed bacterial genomic sequences for many Gram-negative bacteria means that efficient high-throughput techniques will increasingly be needed for functional genomic analysis of these extraordinarily diverse bacteria.

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