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bacterial growth behavior (due to expression of the gene) or the targeted gene is unstable or prone to recombination events in vivo, which significantly reduces targeting efficiencies.

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Gene Replacement in Gram-Negative Bacteria: the pMAKSAC Vectors

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The implementation of targeted modifications in genomes through homologous recombination has become an important and widely used genetic tool. In the case of bacteria, a number of strategies introduce short deletions and insertions, or even entire foreign genes or operons in the chromosome of a given host.

A popular strategy relies on the use of narrow host range plasmids to force the chromosomal recombination of foreign genes in bacteria in which the plasmid is unable to replicate. A second recombination event that leads to the loss of vector sequences is subsequently selected for by the use of the plasmid-encoded *sacB* gene of *Bacillus subtilis*. This gene confers sucrose sen-

sitivity to the host cells (12-16) or the *rpsL* gene from *Escherichia coli*, which confers streptomycin sensitivity to streptomycin-resistant cells (18,19). However, these approaches are not suitable for bacteria in which the plasmid can replicate. To circumvent this problem, conditional replication "suicide" plasmids are used. For example, plasmid pMAK700 expresses a thermosensitive replication initiation protein (repA_{ts}) derived from *ts* mutants of plasmid pSC101, such that replication from its origin occurs at 30°C but not at 42°C (5). In another design, plasmids pJM703 (11) and pKNG101 (6) are driven by the conditional origin of replication of the R6K plasmid (*oriR6K*), which is only functional in the presence of the π protein coded by the *pir* gene.

Under nonpermissive conditions for replication (growth at 42°C for pMAK700, absence of the *pir* gene for pJM703 and pKNG101), the selection for a plasmid-borne marker (e.g., an antibiotic resistance gene) will only allow

Table 1. Sucrose Sensitivity of *E. coli* Cells Carrying *sacB*-Expressing Plasmids^a

Suicide Vector	Medium		
	LB	LB	LBS-N
pMAK700ori	3.0×10^8	3.8×10^8	3.8×10^8
pKNG101	4.6×10^8	4.5×10^3	1.1×10^4
pMAKSACB	6.0×10^8	7.9×10^4	8.0×10^3

^aData are given in CFU/mL.
^bLB: standard medium containing 1% NaCl. LBS: LB with 6% sucrose. LBS-N: LBS without NaCl.

Table 2. Efficiency of Sucrose Selection and Genetic Modification in *V. cholerae*^a

Integration plasmid	Sucrose ^b	Total ^c	Cm ^S (d)	% Cm ^S	Cm ^S Km ^R	%Km ^R in Cm ^S (e)
pMAKHMK-1	-	6.6×10^8	3.2×10^8	48	3.1×10^7	10
pMSHMK-2	-	5.6×10^8	3.7×10^8	66	9.2×10^7	25
	+	3.2×10^8	3.2×10^8	100	1.4×10^8	43

^aData are given in CFU/mL.
^bGrowth on LB (-) or LBS-N (+) with or without added antibiotics.
^cGrowth on LB.
^dDetermined by subtraction of Cm^R colonies (determined from growth on solid LB-Cm) from the total number of colonies.
^ePercent of Km^R colonies among the Cm^S colonies.

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the survival of those cells that have chromosomally integrated the marker. The latter event normally occurs through Campbell-like recombination between homologous regions present on both the chromosome and the plasmid, which results in the integration of the entire plasmid into the chromosome. In most cases, it is desirable to eliminate the vector and the wild-type DNA sequences from the co-integrate molecule. This is possible through a second cross-over event occurring on the other side of the modified DNA with respect to the first cross-over. Because the frequency of such a crossover is rather low, it is often necessary to rely on a positive selection system to detect it. For example,

replication at 30°C from the rep101ts replicon of chromosomally integrated pMAK700 was sometimes found to be toxic to the cell. Therefore integrant cells that grow quickly at 30°C tend to have lost the vector sequences.

Either the wild-type or mutated sequences are conserved in the host chromosome depending on the location of the cross-over. For pKNG101, selection for loss of the vector is achieved through the presence in the vector of the *sacB* gene of *B. subtilis*, which mediates sucrose sensitivity (4). The addition of 5% sucrose in the medium will select for those cells that have lost the vector, including the *sacB* gene, but these techniques are not completely re-

liable. In the course of our work using these plasmids, we have found that, in some cases, over 99% of host cells carrying a co-integrated pMAK700 replicon were still able to grow at 30°C. On the other hand, using pKNG101, we were faced with stability problems that could not be solved.

Blomfield et al. (2) combined sucrose and thermosensitive replication selections for loss of vector sequences, thereby providing a possible solution to these problems. However, they failed to produce a convenient vector for direct use, focusing instead on constructing a *sacB-Neo^R* cassette that could be transferred to other plasmid systems. Link et al. (9) created a similar vector called pKO3 based on the same combination of selections. However, the number of cloning sites available is small, and there is no information about other restriction sites present around the vector. Neither of these vectors allows conjugational transfer to recipient strains.

Because we needed to address these limitations in our own work, we constructed two suicide vectors, named pMAKSACA and pMAKSACB, based on pMAK700 and the *sacB* gene. Their main features are the rep101ts origin of replication, the *sacB* gene and polylinker from pKNG101, an *oriT* sequence for conjugational transfer and the chloramphenicol-resistance gene (*cat*) as a selection marker. About 1.7 kb of unnecessary DNA present in pMAK700 has been removed to limit the size of the resulting vectors and eliminate a number of unwanted restriction sites. An additional advantage of the present vectors is that their nucleotide sequence has been reconstructed from published data and is available on request from the authors.

The construction of pMAKSACB is presented in Figure 1. First, the *oriT* sequence was cloned as a 364 bp *SmaI* fragment (personal communication from M. Alexeyev) from pBSL237 (1) in the unique *EcoRV* site from pMAK700 to produce pMAKoriT. Its orientation could be determined by *NaeI* restriction (result not shown). Second, two unnecessary regions of pMAKoriT, a 742 bp *AatII-StuI* and a 642 bp *HindIII-AgeI* fragment were removed to give pdMAK and pddMAK, respectively. Third, the 333 bp *SphI* and *NheI* fragment from pddMAK was

Figure 1. Construction of pMAKSACB. Black arrow: gene for the temperature-sensitive replication-initiation protein (*repA*ts); open arrow: chloramphenicol-resistance gene (*cat*); MBN: mung bean nuclease. The designations of intermediate plasmid constructs are boxed. Only the most relevant restriction sites are shown. Plasmid coordinates are given in kilobase pairs.

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replaced by the 2497 bp *EcoRV-XbaI* fragment from pKNG101 carrying *sacB* and a polylinker to produce pMAKSACA and pMAKSACB. The orientation of this fragment in pMAKSACA is opposite to that of the *cat* gene, while both genes are in the same orientation in pMAKSACB. Available cloning sites are *ApaI*, *AvrII*, *AsnI*, *BamHI*, *MluI*, *NheI*, *NsiI*, *PstI*, *Sall* and *SmaI*. Note that there are three restriction sites for *PstI* and two for *SmaI*. These are all clustered in an unessential region between *sacB* and the polylinker such that restriction with these enzymes remains possible and can even be useful. *PstI* restriction, for example, removes a 573 bp fragment that includes the unique restriction sites for *BamHI*, *Sall*, *AsnI* and *XmnI*. Therefore these sites become available for modification of the target DNA cloned in *PstI*. Likewise, restriction with *SmaI* removes a 227 bp fragment carrying a unique *MluI* restriction site. Note that *EcoRI* and *HindIII* restriction sites are present at vital locations in the vector (Figure 1). Hence, these restriction enzymes cannot be used for cloning. pMAKSACA and pMAKSACB are shown in Figure 2.

The functional features of pMAKSACA and pMAKSACB were then tested. Both vectors were efficiently transferred from the *E. coli* donor strain S17.1 (17) into either an *E. coli* or a *Vibrio cholerae* recipient strain. In addition, the temperature sensitivity afforded by *rep101ts* prevented any growth at 42°C when approximately 5×10^4 cells or less were plated on a selective medium. Plating of more cells resulted in the appearance of patches of microcolonies that transiently kept the vectors as independent replicons. Published data indicate that in certain cases, the *sacB* selection can be improved by reducing the amount of NaCl in the growth medium (2). Therefore sucrose sensitivity was tested on LB medium (10) containing 6% sucrose (LBS) with or without NaCl (LBS-N).

Table 1 shows the results for the *E. coli* K12 strain DH10B carrying pMAKSACB. DH5 α (pMAK700) and CC118 (pKNG101) were used as negative and positive controls, respectively. Approximately 7.9×10^4 DH10B (pMAKSACB) colony-forming units (CFU)/mL were still produced on

LB medium with 6% LBS at 30°C, which corresponds to an approximate 7×10^3 reduction factor with respect to viable count. This is about 14-fold less than pKNG101, which affords an approximate 1×10^5 reduction factor with respect to viable count. However, when NaCl was omitted from the growth medium (LBS-N) a further reduction to approximately 8.0×10^3 CFU/mL was obtained. This corresponds to an approximate 7×10^4 reduction factor with respect to viable count, bringing the sucrose sensitivity from pMAKSACB to a level similar to that from pKNG101.

Similar results were obtained for pMAKSACA (results not shown).

Next, pMAKSACA was evaluated as an integration suicide vector. As a model system, we wished to integrate the kanamycin-resistance (*Km^r*) gene from Tn903 into the mercury-resistance operon (*mer*) present in the *V. cholerae* vaccine strain CVD103-HgR2 (7). Thus, a 952 bp *SmaI* cassette carrying the *Km^r* determinant was isolated from plasmid p34S-Km3 (3) and inserted at a *StuI* site present downstream of *merR* in plasmid pJMK10 (7). The latter plasmid corresponds to pUC19

Figure 2. Maps of pMAKSACA and pMAKSACB. Open box: region necessary for replication of the *rep101ts* replicon; *oriT*: replication origin of the *rep101ts* replicon. All 6 bp unique class II restriction sites are featured. Also shown are double *SmaI*, triple *PstI* sites and a unique *XmnI* site, which are useful for cloning.

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bearing a 10 kb *Pst*I insert comprised of the *mer* operon inserted within the hemolysin locus of *V. cholerae* (*hly::mer* locus). A 9288 bp *Bam*HI-*Bgl*II fragment carrying the *hly::mer::Km^r* locus, which provided 2654 and 5682 bp of homologous regions adjacent to the *Km^r* gene, was then subcloned in either pMAKSACA or pMAK700 that were cut by *Bam*HI to produce pMSHMK2 and pMAKHKM1, respectively.

The methodology for integration of the plasmids into the chromosome of host strains has been described previously (20). Briefly, the plasmids were transferred by electroporation (pMAKHKM1) or conjugation (pMSHMK2) into CVD103-HgR2, and cells with chromosomally integrated plasmids were selected for by two rounds of growth at 42°C on LB plates containing 17 µg/mL chloramphenicol (LB-Cm). Several colonies pooled from the latter plates were then resuspended in a small volume of LB and the suspension was incubated for about 2 h at 30°C. Suitable dilutions were then plated onto LB or LBS-N agar plates and further incubated at 30°C for 16 h. The colonies were scored for viable count, and the plates were replicated onto LB-Cm, LB with 50 µg/mL kanamycin (LB-Km) and LB plates, in this order. After overnight incubation at 30°C, the chloramphenicol-resistant (Cm^R) and kanamycin-resistant (Km^R) colonies were scored. From these results, the number of Cm-sensitive (Cm^S) and Cm^S Km^R colonies were scored and expressed as CFU/mL.

The results shown in Table 2 indicate that 66% of the pMSHMK2-carrying cells and 48% of the pMAKHKM1-carrying cells have become Cm^S after the 42°C to 30°C temperature shift-down and growth on normal LB medium. When grown on LBS-N, all pMSHMK2 integrants were Cm^S. This result highlights the higher efficiency of the combination of sucrose and thermosensitive replication selections, which relied solely on toxicity due to *rep101ts* replication. It is also more reliable. In the experiment presented, the efficiency of selection by thermosensitivity alone may seem reasonably high. However, we have had cases where the same strategy yielded <1% Cm^S colonies after temperature shift-down. In contrast, the combination of the two

selections has consistently yielded over 95% Cm^S colonies (results not shown).

Finally, the proportion of Cm^S Km^R colonies (i.e., those that have conserved the integrated *Km^r* gene while having lost the vector and wild-type sequences) was lower than the theoretical value of 50%, in the range of 10%–43%. This follows our observation that the implementation of genetic changes is often partially counterselected. In conclusion, we have constructed versatile and efficient suicide replacement vectors that should be useful for the genetic modification of many Gram-negative organisms.

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