1056–1062 *Nucleic Acids Research, 1999, Vol. 27, No. 4*

Random mutagenesis by recombinational capture of PCR products in *Bacillus subtilis* and *Acinetobacter calcoaceticus*

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Received October 9, 1998; Revised and Accepted December 16, 1998

ABSTRACT

We describe a general method for random mutagenesis of cloned genes by error-prone PCR or DNA shuffling that eliminates the need for post-amplification subcloning following each cycle of mutagenesis. This method exploits the highly efficient and recombinogenic nature of DNA uptake during natural transformation in the Gram-positive bacterium *Bacillus subtilis* and the Gram-negative bacterium *Acinetobacter calcoaceticus*. Plasmid systems were designed that allow capture of PCR-amplified DNA fragments by marker-replacement recombination with a structurally similar helper plasmid resident in the transformation recipient. This recombination event simultaneously transfers the amplified sequences into the helper plasmid and restores the integrity of a drug resistance gene, thereby affording a direct selection for fragment capture. Although this strategy was sufficiently effective to permit recovery in *B.subtilis* of up to 10^3 transformants/μg of PCR product, equivalent plasmid systems were ~100 times more efficient in *A.calcoaceticus*. *Acinetobacter calcoaceticus* also offers the advantage of essentially constitutive transformation competence in ordinary complex broth, such as LB, in contrast to two-step growth in semi-synthetic media required for optimal transformation of *B.subtilis*.

INTRODUCTION

Random mutagenesis of cloned genes is of fundamental importance both for functional analysis of uncharacterized open reading frames and for directed improvement or modification of well-characterized genes or gene products. Although many creative approaches have been described for relatively random mutagenesis of DNA segments both *in vitro* and *in vivo* (1–4), PCR-mediated mutagenesis, accomplished by amplification under conditions that increase normal misincorporation errors (5), has emerged as the method of choice for most applications. PCR mutagenesis protocols are simple and substitutions show a high degree of randomness (6,7). Under some circumstances, the power of this approach may be enhanced even further by template fragment-shuffling and amplification-out-crossing of potentially deleterious substitutions, a method referred to as 'sexual PCR' (8). A DNA segment of interest can in principle be subjected to multiple cycles of mutagenesis, out-crossing and phenotypic screening or selection to yield optimized or significantly modified gene function. Indeed, these technologies have revolutionized the field of protein engineering in recent years (9,10).

The most technically demanding aspect of PCR-mediated mutagenesis remains efficient subcloning of amplified products. Recovery of a comprehensive library of substitutions in a mutagenized target may require generating several thousands of transformants and this can be difficult, particularly when conditions needed for error-prone amplification reduce product yield. Although there have been useful technical refinements in PCR product cloning techniques, such as T-A cloning (11), polymerase polishing of PCR product blunt ends (12) and use of esoteric vectors (13), the efficiency of this step remains unpredictable. When a protein engineering application requires multiple cycles of mutagenesis, the DNA manipulation steps can be tedious. An attractive potential solution to this problem is to utilize the natural recombination and repair machinery of the cell to capture PCR products directly, eliminating altogether the need for subcloning manipulations. This kind of approach has been most effectively exploited in *Saccharomyces cerevisiae*, where strategies dependent upon gap-repair recombination have achieved efficiency of PCR product capture sufficient to generate libraries of mutational substitutions (14). Nevertheless, effective use of this approach is technically challenging and requires specialized expertise. In bacterial systems, this kind of approach has been explored less extensively. Attempts to develop recombinational cloning or fragment-capture recombination systems in *Escherichia coli* have met with limited success (15). Plasmid marker-rescue transformation has been widely exploited for recombinational cloning in the naturally transformable Gram-positive species *Bacillus subtilis*, but only in the context of recombination-mediated recovery of cloned fragments ligated to vector arms that provide extensive flanking homology for recombinational interactions with segments present in a 'helper' plasmid resident in the transformation recipient (16,17). The well-known observation that transformation frequency in *B.subtilis* falls sharply as the size

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of transforming DNA decreases may have discouraged efforts to recover PCR products by recombinational cloning (18).

It is surprising that recombinational cloning or plasmid-based fragment-capture recombination systems were never described for the naturally transformable Gram-negative species *Acinetobacter calcoaceticus*. Although the mechanism of DNA uptake and integration in *A.calcoaceticus* has not been well characterized, the process is known to be extraordinarily efficient and highly recombinogenic (19). To date, these properties of the *A.calcoaceticus* system have been exploited only for the transfer of point mutations to the chromosome via homologous recombination (20).

In the present work, we explored the utility of *B.subtilis* and *A.calcoaceticus* for recombinational capture of PCR products and have compared the two organisms directly, using structurally similar donor and helper plasmid recombination cassettes in which recombinational repair of a truncated kanamycin resistance gene provided a direct selection for PCR product capture. With the *B.subtilis* fragment-capture system, we found it difficult to recover $>10^3$ transformants/μg of PCR product, even when donor–helper homology was well over 500 bp on either side of the cloned insert. With the *A.calcoaceticus* system, structurally analogous donor–helper constructions yielded ~100 times more transformants, making recovery of a comprehensive mutational substitution library of up to 10^5 transformants/μg of PCR product readily achievable. We also found that *A.calcoaceticus* cultures were nearly continuously competent for transformation when grown in standard complex media such as LB broth. In contrast, *B.subtilis* requires adherence to a careful two-step growth regimen in semi-synthetic media for optimal transformation competence. As a general system for achieving recombinational capture of PCR products, *A.calcoaceticus* thus offers significant advantages over *S.cerevisiae*, *E.coli* and *B.subtilis*.

MATERIALS AND METHODS

Bacterial strains and transformation

Bacterial strains used were: *E.coli* MM294 (*endA thiA hsdR17 supE44*) (21); *E.coli* DH5α-*mcr* [F$^-$λ$^-$φ80d*lacZ*Δ M15Δ (*lacZYA-argF*)] (Gibco BRL); *B.subtilis* BD170 (*trpC2 thrA5*) (22); *A.calcoaceticus* BD413, obtained from American Type Culture Collection (ATCC 33305) (23). All strains were grown in LB broth at 37°C, unless stated otherwise. When appropriate, LB broth was supplemented with 50 μg/ml kanamycin (Km) or 100 μg/ml ampicillin (Ap) for *E.coli*, 5 μg/ml Km or 5 μg/ml chloramphenicol (Cm) for *B.subtilis* and 15 μg/ml Km or 100 μg/ml Ap for *A.calcoaceticus*.

Competent cultures of *B.subtilis* BD170 containing pHelpBs were obtained by growth at 30°C in the presence of Cm at 5 μg/ml by the conventional two-step procedure (24). For transformation experiments the competent cells (0.2 ml) were incubated with 0.1 μg of DNA for 1 h at 30°C with shaking and plated on LB agar containing 5 μg/ml of Km. Colonies were counted after incubation for 36–48 h at 30°C. The sporulation phenotype of *B.subtilis* cells was determined on DSM agar (25).

Competent cultures of *A.calcoaceticus* BD413 containing pHelpAc were obtained as described (26) using the following modifications. An overnight culture in LB broth containing 100 μg/ml Ap was diluted 1:50 into 50 ml of LB broth with 100 μg/ml Ap and incubated for 3 h at 30°C. Cells were collected by centrifugation (15 min, 3000 *g*), resuspended in 5 ml of LB broth and used for transformation experiments. The competent cells (0.2 ml) were incubated with 0.1 μg of DNA for 1 h at 37°C without shaking and then plated on LB agar containing 15 μg/ml of Km. Colonies were counted after incubation for 36–48 h at 37°C.

Plasmids

DNA manipulations were carried out using standard methods (27). To construct donor plasmid pMutBs (Fig. 2), a 1.49 kb *Pst*I–*Xba*I fragment from pKD102 (a gift from W. Haldenwang), containing the Km resistance gene, *kan*, of pJH1 (28), was initially introduced into the MCS of pUC18 and then isolated as a 1.37 kb *Hin*dIII fragment and cloned into the *Hin*dIII site of *B.subtilis*/*E.coli* shuttle vector pKSV7 (29).

Two DNA fragments were inserted into pMutBs. A 1.8 kb *Bam*HI–*Sac*I DNA fragment of *Arabidopsis thaliana*, encoding the floral poly(A)-binding protein PAB5 (30), was cloned into the MCS of pMutBs to yield pMutBs::Pab5. A 1.7 kb *Bam*HI–*Sac*I DNA fragment of pBG15 (2) containing the promotorless *spo0A* gene of *B.subtilis* was inserted into the MCS of pMutBs to yield pMutBs::Spo0A.

pMutBs was digested by *Eco*RV to delete a 99 bp fragment from the C-terminus of the *kan* gene. A 1025 bp *Eco*RV fragment of pMutBs, which contains the truncated form of the *kan* gene, Δ*kan*, was inserted into the *Sma*I site of pKSV7. The resulting plasmid, carrying Δ*kan* in the same orientation as the *kan* gene in pMutBs, was named pHelpBs (Fig. 2) and used as the transformation helper resident plasmid in *B.subtilis* BD170. The orientation of Δ*kan* in pHelpBs, isolated from *B.subtilis*, was verified by sequencing through the ligation sites.

To obtain donor plasmid pMutAc (Fig. 3), the MCS from pUC18 was inserted as a 56 bp *Eco*RI–*Hin*dIII restriction fragment into the *A.calcoaceticus*/*E.coli* shuttle vector pWH1274 (31). This new plasmid was then digested by *Xba*I and *Sal*I and ligated with a 1496 bp *Xba*I–*Sal*I fragment of pKD102 containing the *kan* gene.

A 1.8 kb *Bam*HI–*Sac*I DNA fragment containing the *pab5* gene was isolated from pMutBs::Pab5 and cloned into the MCS of pMutAc to yield pMutAc::Pab5. A 1.7 kb *Bam*HI–*Sac*I DNA fragment from pMutBs::Spo0A, which containes the promotorless *spo0A* gene of *B.subtilis*, was inserted into the MCS of pMutAc to yield pMutAc::Spo0A.

A 564 bp *Eco*RI–*Sph*I fragment was removed from pWH1274 and replaced by a 1085 bp *Eco*RI–*Sph*I fragment from pHelpBs carrying the Δ*kan* gene (Fig. 2). The resulting plasmid, which contains Δ*kan* in the same orientation as the *kan* gene in pMutAc, was named pHelpAc (Fig. 3) and used as the helper resident plasmid in *A.calcoaceticus* BD413.

PCR and DNA sequencing

PCR reactions were carried out under the following conditions, hereafter referred to as standard: 1 ng of DNA template, PCR buffer (Boehringer Mannheim), 0.2 mM of each dNTP, 0.5 mM of both primers, 1.5 mM $MgCl_2$ and 2.5 U *Taq* DNA polymerase (Boehringer Mannheim) in 0.1 ml volume. PCR reactions were performed in an Ericomp thermocycler (Ericomp Inc.) for 25 cycles: 1 min at 94°C, 40 s at 50°C and 40 s at 72°C, followed by 8 min extension at 72°C. Sequencing and PCR primers were obtained from DNAgency (Aston, PA). The PCR primer sequences used were: 5′-CCCGAAGAGGAACTTGTCTTTTC-3′, K1 (155); 5′-GCATGATGGCTGGAGCAATC-3′, K2 (451); 5′-GCAAG-

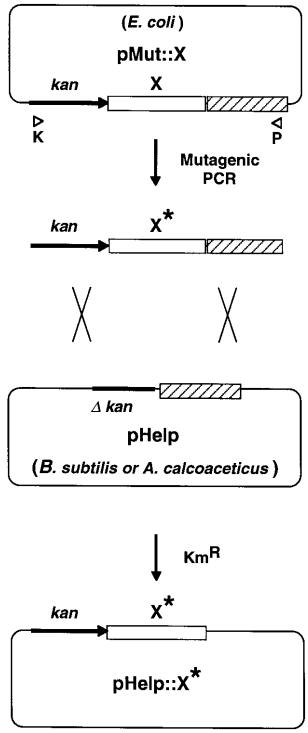

Figure 1. Strategy for recombination-mediated capture of cloned genes mutagenized by error-prone PCR (one round of mutagenesis is shown). Donor plasmid pMut and helper plasmid pHelp are structurally similar chimeric replicons. X, a cloned gene to be mutagenized; *kan*, a Km resistance determinant selectable in *E.coli*, *B.subtilis* and *A.calcoaceticus*; Δ*kan*, truncated form of the *kan* gene; hatched box, a region of homology between pMut and pHelp; X*, cloned gene altered by mutagenic PCR; K and P, PCR primers.

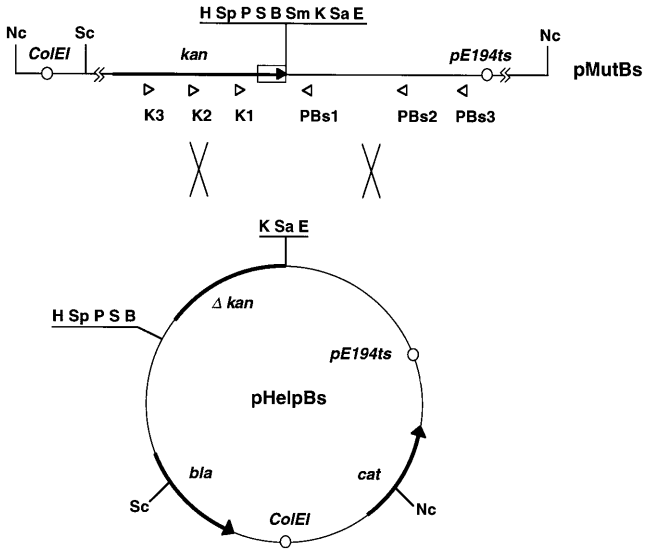

Figure 2. Relevant features of the pMutBs and pHelpBs plasmid pair. Open box represents region of non-homology between plasmid sequences. The location and orientation of plasmid-encoded genes are indicated by arrows. *bla*, β-lactamase gene and *cat*, Cm acetyltransferase gene, derived from the parent plasmid pKSV7; *kan*, the 3′5″-aminoglycoside phosphotransferase III gene; Δ*kan*, truncated form of the *kan* gene. Origins of replication from the parent plasmid pKSV7 are represented by open circles. The restriction sites shown: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; Nc, *Nco*I; P, *Pst*I; S, *Sal*I; Sa, *Sac*I; Sc, *Sca*I; Sm, *Sma*I; Sp, *Sph*I. The *Sph*I and *Hin*dIII sites, shown in pMutBs, are not unique. The positions of the PCR primers are indicated by open arrowheads.

GAACAGTGAATTGGAGTT C-3′, K3 (781); 5′-CATAAGTG-CGGCGACGATAGTC-3′, K4 (1106); 5′-TTAGGCACCCCA-GGCTTTACAC-3′, PBs1 (101); 5′-GTCCAGAGAACAAAC-CTGTACG-3′, PBs2 (610); 5′-CCTCGCAGCACGATATAAA-G-3′, PBs3 (918); 5′-GGCGACACGGAAATGTTGAATAC-3′, PAc1 (250); 5′-CCGTAAGATGCTTTTCTGTGACTGG-3′, PAc2 (561); 5′-CCCAACGATCAAGGCGAGTTAC-3′, PAc3 (713); 5′-GGATTAGCAGAGCGAGGTATGTAGG-3′, PAc4 (1479). The numbers in parentheses after the primer names indicate the size (bp) of flanking homology between the PCR product and pHelp.

Mutagenic PCR reactions were carried out with a reduced concentration of dATP (dGTP:dATP ratio of 5:1) in the presence of 0.5 mM $MnCl_2$ (condition B) or absence of $MnCl_2$ (condition A) as previously reported (5), but without addition of DMSO and β-mercaptoethanol. PCR reaction yield was estimated after agarose gel electrophoresis.

For DNA sequencing, a fmol™ DNA cycle sequencing kit (Promega Co.) and ^{32}P-end-labeled oligonucleotides were used. Sequencing primers spanned both strands of the *spo0A* gene at intervals of ~200 bp.

RESULTS AND DISCUSSION

General strategy

Our general strategy for design of donor and helper plasmids was modeled after the marker-rescue system described previously for *B.subtilis* (17). In our adaptation of this strategy, the donor plasmid contained a cloning site placed very close to a Km resistance gene, *kan*, selectable in both *B.subtilis* and *A.calcoaceticus*. Foreign genes to be subjected to PCR-mutagenesis would be inserted at this site in the donor plasmid and amplified using primers annealing at various distances on either side of the site. Helper plasmids were *B.subtilis* or *A.calcoaceticus* replicons that contained DNA segments structurally similar to the region surrounding the donor cloning site, except that the *kan* gene was truncated by 99 bp, rendering its gene product completely inactive (data not shown). PCR products from the donor plasmid amplified across the cloning site were thus able to recombine with the helper plasmid by double-crossover and to restore integrity of the truncated *kan* gene (Fig. 1). Moreover, every recombination event that restored Km^R would also integrate the cloned gene into the helper plasmid. We refer to the donor plasmid as pMut and the helper plasmid as pHelp; the plasmid pair constructed for *B.subtilis* is pMutBs/pHelpBs and the pair constructed for *A.calcoaceticus* is pMutAc/pHelpAc.

The *B.subtilis* system

Relevant features of pMutBs and pHelpBs are indicated in Figure 2. To evaluate this donor–helper system, two test inserts were cloned into pMutBs. One insert consisted of a 1.7 kb *B.subtilis* chromosomal DNA fragment containing the *spo0A* gene (pMutBs::Spo0A); the other consisted of a 1.8 kb *A.thaliana* chromosomal DNA fragment containing the floral poly(A)-binding protein PAB5 (pMutBs::Pab5). To determine how fragment size, size of insert and extent of homology flanking an insert affected transformation efficiency in this system, restriction digests of pMutBs, pMutBs::Spo0A and pMutBs::Pab5 or PCR products

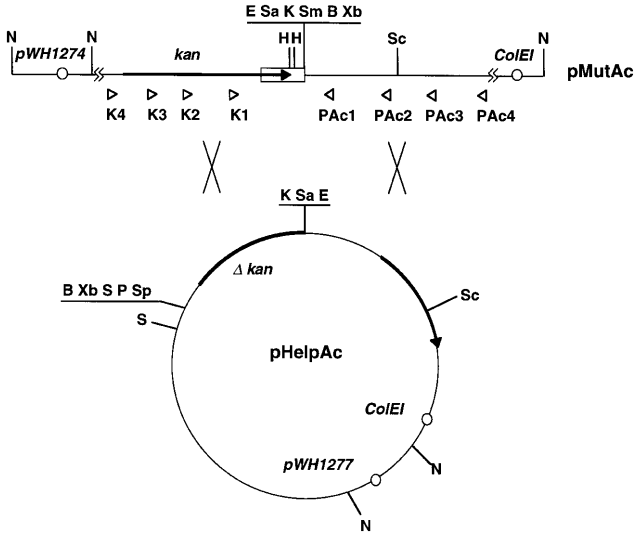

Figure 3. Relevant features of the pMutAc and pHelpAc plasmid pair. Open box represents the region of non-homology between plasmid sequences. The location and orientation of relevant plasmid-encoded genes are indicated by arrows, origins of replication from the parent plasmid pWH1274 are represented by open circles. The restriction sites shown: N, *Nde*I (see also legend to Fig. 2). The positions of the PCR primers are indicated by open arrowheads.

amplified from these plasmids were used to transform *B.subtilis* strain BD170 containing pHelpBs. To minimize the presence of plasmid multimers in samples of pMutBs, pMutBs::Spo0A and pMutBs::Pab5, these plasmids were prepared in a *recA* mutant of *E.coli*; thus, even for uncut plasmid DNA, recovery of transformants was almost completely dependent upon recombinational interaction with pHelpBs (data not shown). As expected, uncut plasmid DNAs were the most biologically active, yielding ~10^5 transformants/μg in each case (Table 1). Although the yield was slightly higher for pMutBs than for pMutBs::Spo0A and pMutBs::Pab5, the presence of inserts apparently made little difference. Double-digestion of pMutBs and pMutBs::Spo0A with *Nco*I and *Sca*I linearized the plasmids and left 1.95 and 3.71 kb of homology with pHelpBs on either side of the insert cloning site. This treatment reduced transformation efficiency by ~10-fold relative to the uncut sample. To estimate the lower limits of transforming fragment size and flanking homology required for biological activity, PCR products were generated from the three plasmid templates using primers annealing at varying distances from the insert cloning site. The primers K3 + PBs3 generated PCR products with homology of 781 and 918 bp to either side of the cloning site for all three amplification templates, but the products contained non-homologous inserts of varying sizes: 164 bp for pMutBs; 1.86 kb for pMutBs::Spo0A; 1.96 kb for pMutBs::Pab5. When comparing transformation efficiencies for the three products it should be noted that they differ from each other significantly both in total size and in the size of the non-homologous insert. It is thus interesting to consider that transformation efficiencies for the three products were not very different: 1.2×10^3/μg for pMutBs; 6.0×10^2/μg for pMutBs::Spo0A; 8.6×10^2/μg for pMutBs::Pab5. This implies that the extent of homologous DNA flanking the insert available for recombinational synapse is more important than insert size or total fragment size, at least in the range of variation tested here. The products generated by amplification with primers K1 + PBs1 yielded almost no transformants above template background. In this case, the homology to either side of the insert was 155 and 101 bp. These results are consistent with the earlier studies (16), which concluded that regions of flanking homology <200 bp are not sufficient to promote effective marker-rescue recombination in *B.subtilis*.

Table 1. Efficiency of recombination-mediated transformation in *B.subtilis* BD170 [pHelpBs]

DNA (template)	Enzymes used	Primers	Efficiency of transformation[a]
pMutBs	None	None	1.4×10^5
	*Nco*I + *Sca*I	None	1.6×10^4
		K3 + PBs3	1.2×10^3
		K2 + PBs2	3.2×10^2
		K1 + PBs1	0.4×10^2
pMutBs::Pab5	None	None	9.2×10^4
		K3 + PBs3	8.6×10^2
		K2 + PBs2	2.4×10^2
		K1 + PBs1	0.2×10^2
pMutBs::Spo0A	None	None	1.0×10^5
	*Nco*I + *Sca*I	None	1.1×10^4
		K3 + PBs3	6.0×10^2
		K2 + PBs2	2.2×10^2
		K1 + PBs1	0.5×10^2

[a]The efficiency of transformation was calculated as the number of Km^R colonies/μg DNA and corrected for the template background, which was determined by transforming the template (1 ng) after mock amplification without primers. Each transformation was repeated at least three times and the average number of Km^R colonies is shown.

The *A.calcoaceticus* system

To compare as directly as possible the efficiency of marker-rescue recombination in *B.subtilis* and *A.calcoaceticus*, we constructed donor and helper plasmids pMutAc and pHelpAc (Fig. 3), which contain a recombination-selection cassette very similar in structure to that described for pMutBs and pHelpBs. The same test inserts examined with the *B.subtilis* system were cloned into pMutAc, to generate pMutAc::Spo0A and pMutAc::Pab5. *Acinetobacter calcoaceticus* replication functions for the *E.coli*/*A.calcoaceticus* plasmid pHelpAc were derived from pWH1274 (31). The copy number of pHelpAc in *A.calcoaceticus* was estimated to be 5–10/cell, approximately the same as the copy number of pHelpBs in *B.subtilis* (data not shown).

The *A.calcoaceticus* BD413 strain was chosen as host for pHelpAc, a strain reported to be highly competent for natural transformation throughout growth and early entry into stationary phase (23). In agreement with observations of others (26), we were able to establish a very simple growth regimen in LB broth for *A.calcoaceticus* BD413 that reproducibly supported a high level of transformation. Also in agreement with previous work (26), we found that transformants of plasmid-free *A.calcoaceticus* BD413 with pMutAc, pMutAc::Spo0A or pMutAc::Pab5 DNA prepared in a *recA* mutant of *E.coli* was nearly as efficient as transformation of *A.calcoaceticus* BD413 containing pHelpAc (data not shown), indicating that transformation of *A.calcoaceticus* is not dependent on plasmid multimers.

To test the efficiency of recombination-mediated fragment capture in *A.calcoaceticus*, pMutAc, pMutAc::Spo0A and pMutAc::Pab5 were digested with *Nde*I. This treatment linearized the plasmids to produce fragments with 2.35 and 3.67 kb of homology on either side of the insert cloning site. The restriction digests showed somewhat reduced efficiency of transformation, relative to the intact plasmid, but still yielded >10^6 transformants/μg (Table 2). As observed with the *B.subtilis* system, digests of the three plasmids yielded comparable transformation frequencies, indicating that insert size had a negligible influence on transformation efficiency, at least for inserts <2.0 kb. As also observed with the *B.subtilis* system, transformation with fragments having progressively limited homology flanking the insert cloning site, whether produced by digestion with restriction enzymes or by PCR amplification with primers annealing closer to the insert, yielded progressively reduced transformation efficiencies. Overall, the *B.subtilis* and *A.calcoaceticus* systems showed similar characteristics qualitatively with respect to transformation efficiency as a function of transforming fragment size, size of non-homologous insert and extent of homology flanking the insert. In quantitative terms, however, the *A.calcoaceticus* system was far superior. For example, the PCR product amplified from pMutAc::Spo0A with primer pair K2 + PAc2 yielded 1.7×10^4 transformants/μg in the *A.calcoaceticus* system, whereas the product amplified from pMutBs::Spo0A with primer pair K2 + PBs2 yielded only 2.2×10^2 transformants/μg in the *B.subtilis* system. Generally, for fragments of similar size, with identical non-homologous inserts and comparable extents of fragment–helper homology flanking the inserts, we observed transformation frequencies in *A.calcoaceticus* ~100 times greater than in *B.subtilis*.

Table 2. Efficiency of recombination-mediated transformation in *A.calcoaceticus* BD413 [pHelpBs]

DNA (template)	Enzymes used	Primers	Efficiency of transformation[a]
pMutAc	*Nde*I	None	3.2×10^6
		K3 + PAc3	2.0×10^5
		K2 + PAc2	3.4×10^4
pMutAc::Pab5	*Nde*I	None	2.0×10^6
		K3 + PBs3	1.0×10^5
		K2 + PBs2	1.7×10^4
pMutAc::Spo0A	*Nde*I	None	2.2×10^6
		K4 + PAc4	4.0×10^5
		K3 + PAc3	1.1×10^5
		K2 + PAc2	1.8×10^4
		K1 + PAc1	1.4×10^3

[a]Described in footnote to Table 1. The background of transformation with restriction fragments was determined by transforming them into plasmid-free *A.calcoaceticus* BD413 and corrected for the difference in competency between the strains.

Recombinational capture of PCR-mutagenized fragments

To evaluate the performance of the *A.calcoaceticus* system in the context of an actual PCR mutagenesis experiment, the *spo0A*-containing insert in pMutAc::Spo0A was subjected to error-prone PCR amplification under conditions of reduced dATP in the absence (condition A) or presence (condition B) of 0.5 mM $MnCl_2$, as described previously (5). Two pairs of primers were used (K3 + PAc3 or K4 + PAc4) and PCR yields were estimated after agarose gel electrophoresis. Amplification under mutagenic conditions, particularly in the presence of 0.5 mM $MnCl_2$, reduced the yield of PCR product slightly (<2-fold), but reduced the yield of transformants somewhat more (>2-fold for condition A; >4-fold for condition B), perhaps reflecting the presence of mutations inactivating the *kan* gene (Table 3). Nevertheless, the yield of transformants was well over 10^4 transformants/μg, even for amplifications with primer pair K3 + PAc3 under condition B. For amplification with primer pair K4 + PAc4 under condition A or B, the yield of transformants was ~10^5/μg (mutation libraries A and B). Four clones from each library were picked to determine the actual mutation rate under these conditions. To detect substitutions unambiguously, the entire *spo0A* coding sequence from each clone was sequenced from both strands. Condition A resulted in a substitution rate of 0.4% (13/3240) and condition B a rate of 1.3% (41/3240), slightly lower than levels reported previously (5). The spectrum of mutations produced is summarized in Table 4. Overall, the distribution and types of substitutions were nearly random, consistent with the results of published studies (6,7).

Table 3. Effect of PCR conditions on the transformation efficiency in *A.calcoaceticus* BD413 [pHelpBs]

PCR condition	DNA yield (μg)[a]	Primers	Efficiency of transformation[b]
Standard	0.7 ± 0.1	K4 + PAc4	4.0×10^6
A	0.5 ± 0.1		1.4×10^5
B	0.3 ± 0.1		8.6×10^4
Standard	0.9 ± 0.1	K3 + PAc3	1.1×10^5
A	0.7 ± 0.1		4.6×10^4
B	0.4 ± 0.1		1.4×10^4

[a]The yield of DNA (μg) per 0.1 ml of the PCR reaction is shown.
[b]Described in footnote to Table 1.

Integration-mediated transfer of mutations to the *B.subtilis* chromosome

We anticipated that transformation of *B.subtilis* with pHelpAc::Spo0A would yield Km^R transformants only by Campbell recombination between the cloned *spo0A* insert and homologous sequences in the chromosome. This expectation was confirmed by demonstration that transformation of *B.subtilis* with pMutAc or pMutAc::PAB5 yielded no transformants at all, whereas pMutAc::Spo0A yielded ~10^3 transformants/μg. Because the *spo0A* insert does not include the promoter, this recombinational exchange would frequently transfer mutations to the single expressed copy of *spo0A*, enabling the detection of recessive mutant alleles. To test this strategy, >10^3 transformants from mutation libraries A and B were pooled to prepare plasmid DNA (libraries A and B were pooled separately) and pooled plasmids were passaged through a *recA*$^+$ *E.coli* strain (MM294) prior to transformation into *B.subtilis*. Such plasmid preparations yielded >10^4 transformants/μg in *B.subtilis*. Because the *spo0A* gene is required for sporulation in *B.subtilis*, null alleles cause an asporogenic (Spo^-) phenotype, which is readily recognized by colony morphology on DSM agar (25). When transformants from library A were tested on DSM, 0.3% (7/2480) were found to be Spo^-; when transformants from library B were tested, 1.2% (11/920) were Spo^-. Although we have no firm basis for estimating

the percentage of substitutions in the *spo0A* coding sequence that would result in the null phenotype, these results clearly establish the principle that mutations captured on a cloned fragment might be screened for phenotypic consequences after allelic exchange with a wild-type chromosomal gene (loss-of-function and recessive alleles) as well as in autonomously replicating plasmids (gain-of-function and dominant alleles).

Performance parameters for PCR mutagenesis

In evaluating the significance of the 100-fold higher efficiency of the *A.calcoaceticus* fragment-capture system over the equivalent *B.subtilis* system it is useful to consider performance requirements for a typical mutagenesis experiment. If the goal is to produce a library of substitutions in a 1.0 kb interval of DNA that includes mutations at each position with a confidence level >90%, then 10^4–10^5 clones serves as a good approximation for the size of the substitution library required (32). The number of mutated clones can be smaller if multiple substitutions are present in each clone, but multiple substitutions increase the possibility that deleterious mutations will mask the effect of favorable mutations or that nonsense mutations will mask the effect of missense mutations. Referring to the data in Table 1, where amplification of pMutBs::Pab5 or pMutBs::Spo0A even with primers resulting in greatest insert flanking homology still yields $<10^3$ transformants/μg, it is apparent that a comprehensive library of substitutions would require a very large scale transformation experiment. In contrast, the *A.calcoaceticus* system would be expected to yield 10^5 transformants/μg (Table 2), easily within the parameters of a small scale experiment.

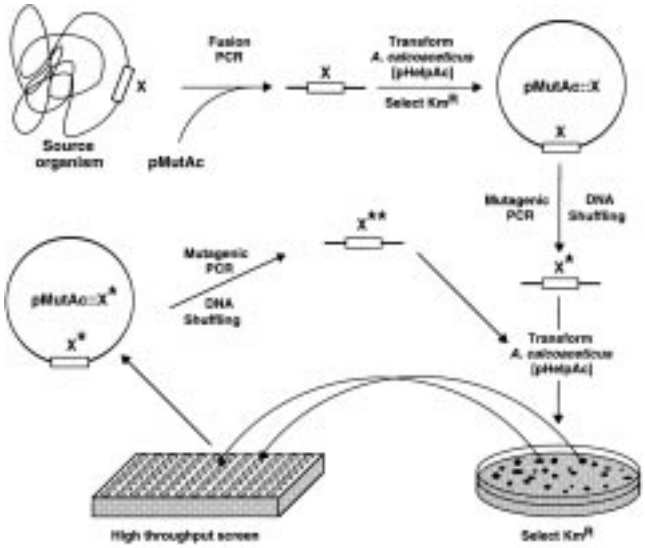

Figure 4. Retrieval and mutagenesis of a gene of interest (X), with multiple cycles of enrichment or screening for improved function, in the complete absence of conventional DNA manipulations. 'Fusion PCR' refers to the overlap extension method of joining together heterologous PCR products amplified from different templates (34). By this method, DNA sequences flanking the insert cloning site in pMutAc would be joined to either side of the gene of interest, providing flanking homology for fragment-capture recombination with pHelpAc. The product of recombination (pMutAc::X) would then serve as the template for mutagenic PCR or DNA shuffling. Amplification products would again be captured by transformation of a pHelp-containing strain, to generate clones for phenotypic screening. Plasmids isolated from selected clones can be used directly for additional cycles of mutagenesis and screening, without the need for post-amplification subcloning.

Applicability of the *A.calcoaceticus* system to multiple cycles of mutagenesis

Although the advantages of the *A.calcoaceticus* system are apparent even in the context of a single round of mutagenesis, these advantages are compounded in an application that requires multiple rounds of mutagenesis. For example, a protein engineering application might involve several cycles of PCR mutagenesis and/or DNA shuffling, with each cycle followed by a high throughput screen for variants with improved stability or catalytic properties. Because the recombination-mediated fragment-capture system described here converts the pHelpAc helper plasmid to Km^R as the cloned fragment is captured, the product of recombination may be used directly for another round of PCR mutagenesis or DNA shuffling without *in vitro* recombinant DNA manipulations of any kind. Indeed, the original cloned fragment that serves as the target for the first round of mutagenesis might itself be captured by recombinational cloning. Thus, as illustrated in Figure 4, a gene of interest might be recovered from the genome of the source organism and subjected to an unlimited number of PCR mutagenesis cycles and high throughput phenotypic screens without ever requiring the use of restriction enzymes or DNA ligase.

Table 4. Effect of PCR conditions on the frequency of mutations in the *spo0A* gene

Mutations	Condition A		Condition B	
	Number of occurrences	Frequency (mutations/bp)[a]	Number of occurrences	Frequency (mutations/bp)[a]
Transitions	8 (61.6%)	2.5×10^{-4}	25 (61.0%)	7.7×10^{-3}
A:T→G:C	4 (30.8%)	1.2×10^{-3}	14 (34.1%)	4.3×10^{-3}
T:A→C:G	2 (15.4%)	6.2×10^{-4}	4 (9.7%)	1.2×10^{-3}
C:G→T:A	1 (7.7%)	3.1×10^{-4}	4 (9.7%)	1.2×10^{-3}
G:C→A:T	1 (7.7%)	3.1×10^{-4}	3 (7.3%)	9.3×10^{-4}
Transversions	4 (30.8%)	1.2×10^{-3}	16 (39.0%)	4.3×10^{-3}
Insertions/deletions	1 (7.7%)	3.1×10^{-4}	0	0
All mutations	13 (100%)	4.0×10^{-3}	41 (100%)	1.3×10^{-2}

[a]A total of 3240 bp were sequenced from both strands of the *spo0A* gene for each PCR condition.

SUMMARY AND CONCLUSIONS

The natural transformation systems of *B.subtilis* and *A.calcoaceticus* can both be exploited for recombinational capture of PCR products, but the *A.calcoaceticus* system is ~100 times more efficient. For applications that demand generating a mutation library containing of the order of 10^4 substitutions, the significantly greater efficiency of the *A.calcoaceticus* system would be an important advantage, even for a single round of mutagenesis.

A further advantage of the *A.calcoaceticus* system is the extraordinary simplicity of its use. Most naturally transformable bacteria are maximally competent for DNA uptake only when grown under special conditions or they acquire transformation competence only at a specific stage of growth, sometimes reflecting the need to accumulate extracellular signalling molecules to a critical concentration (33). For example, the optimal competence regimen for *B.subtilis* involves successive growth of the bacteria in two different semi-synthetic growth media (24). In contrast, a highly transformation-competent preparation of *A.calcoaceticus* BD413 can be obtained simply by dilution of an overnight culture into fresh LB broth and harvesting bacteria at almost any time in mid to late log phase.

Efficiency of fragment capture in both the *B.subtilis* and the *A.calcoaceticus* systems is a sensitive function of the extent of homology flanking non-homologous inserts and it diminishes rapidly when segments of flanking homology are <0.4 kb. The efficiency of fragment-capture is relatively insensitive in both systems to the size of non-homologous inserts, at least for inserts <2.0 kb. The significantly greater efficiency of *B.subtilis* relative to *E.coli* in executing fragment-capture recombination is presumably a reflection of the nature of the DNA uptake process in *B.subtilis*, in which transforming molecules are nicked and converted into the single-stranded form and are therefore highly recombinogenic (18). It is not known whether the DNA uptake process in *A.calcoaceticus* is similar in this respect, but it is interesting to note that the overall characteristics of the *B.subtilis* and *A.calcoaceticus* systems are quite similar qualitatively.

As indicated in Figure 4, the *A.calcoaceticus* system described here not only allows multiple rounds of PCR mutagenesis or DNA shuffling without subcloning, it also permits primary recovery of the potential mutagenesis or protein engineering target from the source organism without cloning, providing that the DNA sequence surrounding the target gene is known. With extensive genome sequencing information available in the near future, it should routinely be possible to scan genome sequence databases for genes of potential interest, retrieve them for functional analysis and use them as the starting points for mutagenesis or as inputs for 'family shuffling' (9) without the need for conventional recombinant DNA manipulations.

ACKNOWLEDGEMENTS

We thank D. Belostotsky and R. Meagher for cloned DNA containing the *A.thaliana pab5* gene and E. Neidle for a sample of *A.calcoaceticus* strain BD413. This work was supported by Public Health Services grant GM 35495 from the National Institutes of Health.

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