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Combining Localized PCR Mutagenesis and Natural Transformation in Direct Genetic Analysis of a Transcriptional Regulator Gene, *pobR*†

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We present a procedure for efficient random mutagenesis of selected genes in a bacterial chromosome. The method combines PCR replication errors with the uptake of PCR-amplified DNA via natural transformation. Cloning of PCR fragments is not required, since mutations are transferred directly to the chromosome via homologous recombination. Random mutations were introduced into the *Acinetobacter* chromosomal *pobR* gene encoding the transcriptional activator of *pobA*, the structural gene for 4-hydroxybenzoate 3-hydroxylase. Mutant strains with strongly reduced PobR activity were selected by demanding the inability to convert 4-hydroxybenzoate to a toxic metabolite. Of spontaneous *pobR* mutants, 80% carry the insertion element IS*1236*, rendering them inappropriate for structure-function studies. Transformation with *Taq*-amplified *pobR* DNA increased the mutation frequency 240-fold and reduced the proportion of IS*1236* inserts to undetectable levels. The relative fidelity of Pfu polymerase compared with *Taq* polymerase was illustrated by a reduced effect on the mutation frequency; a procedure for rapid assessment of relative polymerase fidelity in PCR follows from this observation. Over 150 independent mutations were localized by transformation with DNA fragments containing nested deletions of wild-type *pobR*. Sequence analysis of 89 of the mutant *pobR* alleles showed that the mutations were predominantly single-nucleotide substitutions broadly distributed within *pobR*. Promoter mutations were recovered, as were two mutations that are likely to block *pobR* translation. One-third of the recovered mutations conferred a leaky or temperature-sensitive phenotype, whereas the remaining null mutations completely blocked growth with 4-hydroxybenzoate. Strains containing two different nonsense mutations in *pobR* were transformed with PCR-amplified DNA to identify permissible codon substitutions. Independently, second-site suppressor mutations were recovered within *pcaG*, another member of the supraoperonic *pca-qui-pob* cluster on the *Acinetobacter* chromosome. This shows that combining PCR mutagenesis with natural transformation is of general utility.

Amplification of DNA by thermostable polymerases in the PCR introduces mutations (2, 23, 34, 36, 41). This is an inconvenience when the fidelity of replication is a concern, but in some cases, the randomness of nucleotide substitutions introduced by PCR can facilitate genetic analysis. For example, defects caused by a wide spectrum of independent mutations within a gene can give insight into how structure influences the function of the encoded protein. The efficiency of such structure-function studies is determined in large part by the ease with which the phenotype conferred by altered DNA can be observed. In this regard, oxygenative pathways for dissimilation of aromatic compounds by *Acinetobacter calcoaceticus* (18) provide a convenient genetic system allowing ready selection of defective genes (4, 8, 13) introduced by natural transformation (18).

An intriguing target for genetic investigation is *Acinetobacter* PobR, a member of a family of transcriptional regulators (3) that includes IclR in *Escherichia coli* (35) and *Salmonella typhimurium* (25), GylR in *Streptomyces coelicolor* (33), and KdgR in *Erwinia chrysanthemi* (15). Members of this group are sparse among known transcriptional regulators but are strongly represented among genes governing 4-hydroxybenzoate catabolism in bacteria. These transcriptional regulators govern different structural genes and respond to chemically distinct inducer metabolites: 4-hydroxybenzoate (PobR [3, 5]) and protocatechuate (PcaU [9]), both in *Acinetobacter*, and β-ketoadipate (PcaR [14, 31]) in *Pseudomonas putida* and *Agrobacterium tumefaciens* (30). The causes of the specificity of these different activators and the mechanisms that may be shared by all of them are not yet understood. Attention was directed to *pobR* because strains containing mutations in this gene can be selected readily (3) and the mutations can be mapped precisely with the *Acinetobacter* natural transformation system (3, 4). However, preliminary studies demonstrated that spontaneous *pobR* mutations are caused predominantly by integration of the insertion sequence IS*1236* (10). The genetic basis for preferential insertion of IS*1236* into *pobR* presents an interesting question, but the resulting mutations are of little value for investigation of structure-function relationships in PobR.

The *Acinetobacter* natural transformation system (16, 18, 28, 29) accepts PCR-amplified homologous fragments as donor DNA. Because such fragments are likely to be enriched in mutations caused by single-nucleotide substitutions, mutagenesis by combining PCR amplification and natural transformation offered a promising approach for both selection and characterization of strains in which PobR had been inactivated by a range of single-amino-acid substitutions. Furthermore, the background of spontaneous mutations caused by IS*1236* provided a measure against which the acquisition of PCR-generated nucleotide substitutions could be assessed. Thus we are able to document here a procedure that allows both swift

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assessment and convenient analysis of a wide range of mutations caused by errors introduced by PCR. A major advantage is that the mutations produced are nonpolar, and their physiological consequences can be analyzed directly in vivo. The technique is of general interest because it may be widely applicable in mutagenesis of other chromosomal genes in *Acinetobacter* and in other naturally transformable organisms. In addition, it allows a simple overnight measure of the relative fidelity of PCR amplification.

MATERIALS AND METHODS

Strain and culture conditions. The mineral medium described by Juni (17), supplemented with 10 mM succinate, was routinely used for growth of *Acinetobacter* strain BD413 (16, 18) (designated ADP1 in our laboratory), ADP230 (13), and derivatives in tubes on a gyratory shaker, in 250-μl wells in microtiter plates, or on plates (solidified with 1.8% [wt/vol] agar), at 37°C. Where indicated, 4-hydroxybenzoate (5 mM), quinate (5 mM), or protocatechuate (3 mM) was used as the carbon and energy source.

Recombinant DNA techniques. All recombinant DNA techniques were performed as described previously (21, 22) and according to Sambrook et al. (32). Isolation of bacterial chromosomal DNA, to be used as template DNA in PCRs with *Taq* polymerase, was done with Instagene DNA Purification Matrix (Bio-Rad) according to the protocol provided by the supplier. Instagene preparations of chromosomal DNA did not yield amplification product with Pfu polymerase. Therefore, a scaled-down version of the protocol described before (8) was used for isolation of chromosomal template DNA to be used with Pfu polymerase. Restriction enzymes were obtained from New England Biolabs, Inc. PCR primers were custom synthesized (Keck Biotechnology Resource Laboratory, Yale University).

Transformation of *Acinetobacter*. Strains of *Acinetobacter* were transformed essentially according to the protocol described by Palmen et al. (28), based upon the property that induction of competence for natural transformation takes place at the onset of exponential growth. Routinely, a fresh overnight culture, grown in mineral medium with 10 mM succinate as the carbon and energy source, was diluted 25-fold and grown for 2 h at 37°C in a gyratory shaker. Subsequently, between 500 ng and 1 μg of PCR DNA was added to 500 μl of the fresh culture, which was then incubated for another 3 h. Proper dilutions of the transformation mixture were plated directly onto selective medium or onto nonselective medium for determination of viable counts. Transformation or mutation frequencies are expressed as number of transformants or mutants per 10^6 CFU. For selection of spontaneous mutants, the same protocol was followed, except that no DNA was added.

PCR for transformation-facilitated mutagenesis. *Taq* polymerase (Boehringer Mannheim) and Pfu polymerase (Stratagene) were used as indicated by the suppliers for amplification of DNA fragments. Standard PCRs were carried out with 200 nM (each) primer, 200 μM (each) deoxynucleoside triphosphate, 50 to 100 ng of chromosomal template DNA, and 0.5 U of polymerase in a final volume of 50 μl. The standard thermocycle protocol consisted of a total of 35 cycles, with a denaturation step at 94°C, primer annealing at 58°C (all primers), and elongation at 72°C. Unincorporated primers and deoxynucleoside triphosphates were removed from PCRs with GeneClean Glassmilk as described by the supplier (BIO 101, Inc.).

Generation and mapping of mutations in *pobR*. Mutations that negatively affect *pob* expression (resulting in significant reduction of PobA activity) can be directly selected for in strain ADP230 (Δ*pcaBDK1* [13]) by the procedure outlined in Fig. 1A. After transformation with amplified *pobR* DNA, mutant strains were selected on mineral agar medium with 10 mM succinate (carbon and energy source) supplemented with 5 mM 4-hydroxybenzoate. The Δ*pcaBDK1* deletion in these mutants was restored to wild type by transformation with plasmid pZR3 (12) and selection for growth on quinate. The resulting strains were tested with respect to their ability to utilize 4-hydroxybenzoate as the sole carbon source at both 22 and 37°C.

Mutations in *pobR* and in the *pobA-pobR* intergenic region were mapped in 243 strains by marker rescue, with nested deletion fragments of the wild-type *pobA-pobR* region (see Fig. 3) as donor DNA in transformation (1, 4). For these marker rescue experiments, cells were grown to competence in microtiter plates, and competent cells were transferred directly to mineral medium with 5 mM 4-hydroxybenzoate as the sole carbon source. Prior to the transfer of cells to the selective medium, 500 ng to 1 μg of DNA, carrying a nested deletion fragment of the *Acinetobacter* wild-type *pob* region, was spread onto the plates.

Sequence analysis of mutations. The region of interest for sequence analysis was amplified by PCR with *Taq* polymerase via the standard procedure (except that the concentration of each primer was reduced to 40 nM), this time with chromosomal DNA from the mutant strains as the template DNA. Without further purification, 200 to 300 ng of the PCR DNA was directly used as template DNA in cycle sequence reactions, with the ABI PRISM Dye terminator cycle sequencing kit with Amplitaq DNA polymerase (−FS) as recommended by the supplier (Perkin-Elmer). Cycle sequence products were precipitated at −70°C with ethanol and sodium acetate (pH 4.8) and pelleted in a microcentrifuge at maximum speed. Pellets were washed once with 200 μl of ice-cold 70% (vol/vol) ethanol, air dried for 15 min, and resuspended in a 5:1 (vol/vol) mixture of deionized formamide and 10 mM EDTA (pH 8.0) buffer. DNA fragments were denatured at 95°C for 2 min prior to electrophoresis on a denaturing 6% polyacrylamide gel in an ABI 373 automated sequencer (Perkin-Elmer ABI) linked to an Apple PowerMac equipped with the appropriate sequencing software (Perkin-Elmer ABI). Sequences were analyzed with the DNA analysis program package DNASTAR (Lasergene).

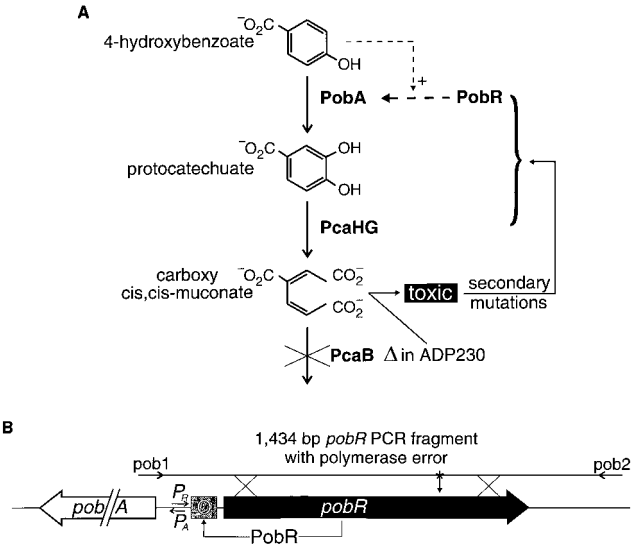

FIG. 1. (A) Selection of mutations in the initial steps in degradation of 4-hydroxybenzoate in *Acinetobacter* strain ADP230 (Δ*pcaBDK1*). PobR positively (+) regulates transcription of the *pobA* gene encoding 4-hydroxybenzoate 3-hydroxylase (PobA), in response to binding 4-hydroxybenzoate as an effector (small dashed arrow [3, 5]). Because of the absence of PcaB, growth in the presence of 4-hydroxybenzoate is only observed in derivatives of ADP230 in which a secondary mutation prevents the accumulation of β-carboxy *cis*,*cis* muconate to toxic levels. (B) Schematic representation of the chromosomal organization of *Acinetobacter pobA* and *pobR*. Small arrows in the top line indicate the primers pob1 (5′-GCAGTTGACCGAGTAGTAATCCCG-3′) and pob2 (5′-GAAAAC TGTCCACTCCGATTCC-3′) used to amplify a 1,434-bp PCR fragment containing 69 bp of *pobA*, the 134-bp *pobA-pobR* intergenic region, the 816-bp *pobR*, and 415 bp of DNA downstream from *pobR*. The star accompanied by a vertical arrow indicates a symbolic mutation generated during PCR amplification of the DNA fragment, and large X's show recombinational events required for assimilation of the mutated fragment into the chromosome after natural transformation. The *pobA* (P_A) and *pobR* (P_R) promoters in the intergenic region are depicted with small arrows; the grey box indicates the operator (O) region to which PobR binds (5). The intergenic regions is presented in greater detail in Fig. 4.

RESULTS

Targeting random mutations to chromosomal genes and rapid assessment of relative polymerase fidelity in PCR. Catabolism of 4-hydroxybenzoate is initiated in *Acinetobacter* by consecutive reactions mediated by 4-hydroxybenzoate 3-hydroxylase (PobA), protocatechuate 3,4-dioxygenase (an oligomer formed by PcaH and PcaG), and carboxymuconate lactonizing enzyme (PcaB [Fig. 1A]). Metabolic accumulation of β-carboxy *cis*,*cis* muconate appears to be toxic, because cells blocked in *pcaB* are unable to grow with succinate in the presence of 4-hydroxybenzoate. Demand for growth on plates containing such medium produces strains containing secondary mutations blocking *pcaH* and *pcaG* (8), *pobA* (13), or *pobR* (the transcriptional activator required for *pobA* expression) (3, 5) (Fig. 1A). Preliminary indication of the site of the secondary mutation can be gained by examining the growth properties of the selected organisms: unlike strains that acquired mutations blocking *pobA* or *pobR*, cells blocked in *pcaH* and *pcaG* can grow in the presence of quinate or protocatechuate.

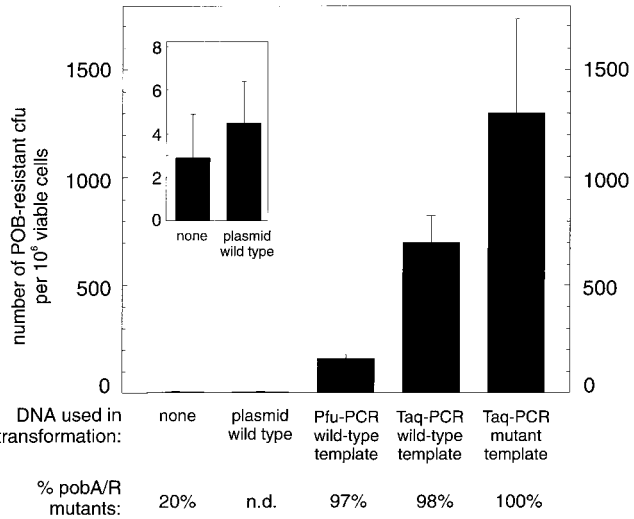

FIG. 2. Measurement of the chromosomal recovery of mutations caused by PCR amplification of *pobR*. In each transformation, identical recipient cultures of strain ADP230 were independently exposed to 500 ng of *pobR* DNA. The percentage of *pobA* or *pobR* mutants among strains selected for resistance to 4-hydroxybenzoate (DOB) is shown below the relevant bar in the graph; n.d., value not determined for cells exposed to plasmid DNA; *n*, number of independent experiments. The frequency of mutants is presented as a fraction of total CFU that resist the toxic effect of 4-hydroxybenzoate in succinate plates (Fig. 1A). Observed values ($\pm\sigma_n$) multiplied by 10^6 were 2.9 ± 2.0 ($n = 4$; no transforming DNA), 4.5 ± 1.8 ($n = 4$; linearized plasmid pZR405), 160 ± 22 ($n = 4$; Pfu-amplified wild-type *pobR*), 700 ± 120 ($n = 3$; *Taq*-amplified wild-type DNA), and 1,300 ± 450 ($n = 2$; *Taq*-amplified mutant *pobR*).

After growth with succinate, 3 in 10^6 of the CFU in a typical culture of *Acinetobacter* strain ADP230 (Δ*pcaBDK1*) are able to grow in the presence of 4-hydroxybenzoate (Fig. 2, [insert]). As judged by sensitivity to protocatechuate, only 20% of these secondary mutants are blocked in either *pobR* or *pobA* (Fig. 2, lower section). The frequency of spontaneous secondary mutations was not significantly affected by transformation of strain ADP230 with plasmid DNA carrying wild-type *pobR* sequences (Fig. 2, insert). This indicates that recombination activity in a specific chromosomal region alone does not detectably influence the mutation frequency. In contrast, exposure of an exponentially growing succinate culture of ADP230 to *Taq*-amplified *pobR* DNA (Fig. 1B) caused a significant 240-fold increase in the frequency of CFU that resist 4-hydroxybenzoate (Fig. 2). As visualized in Fig. 2 (lower section) and described in greater detail below, virtually all of these strains contain mutations blocking *pobR*. This shows that transformation-facilitated PCR mutagenesis is a highly efficient method for targeting mutations to specific chromosomal loci in naturally transformable organisms such as *Acinetobacter* strain BD413 and derivatives.

Successful chromosomal recovery of DNA that had undergone mutagenesis by PCR amplification suggested that the transformation assay described above could provide a relative measure of the mutation frequency in a population of amplified DNA molecules. In other words, a swift assessment of relative fidelity of PCR amplification could be obtained via natural transformation. To illustrate this procedure, we have compared *Taq* polymerase as an enzyme with relatively high error frequency in PCR (36) with the less error-prone Pfu polymerase (24) (Fig. 2). The lower limit of measurement can be derived from the mutation frequency after transformation of *Acinetobacter* strain ADP230 with wild-type *pobR* DNA on a plasmid that has replicated in *E. coli* in the presence of repair enzymes in vivo. As already described, exposure of ADP230 cells to this DNA did not alter the frequency of mutants in the recombinant population from that observed in a population of cells that had not encountered donor DNA. The upper limit of measurement was assessed by exposure of cells to the same amount of DNA amplified with *Taq* polymerase from a *pobR* null mutant; this provided a measure of the greatest number of transformants that it was possible to obtain with DNA of the size and concentration used. As shown in Fig. 2, DNA amplified from the null mutant allowed selection of recombinants with a frequency 500-fold higher than that observed with plasmid-derived *pobR* DNA and 2-fold higher than that observed with DNA that had been amplified from wild-type *pobR* with *Taq* polymerase. Amplification of wild-type *pobR* with the higher-fidelity Pfu polymerase produced DNA that gave rise to *pobR*-deficient recombinants with a frequency about fivefold lower than that observed with DNA amplified from wild-type *pobR* with *Taq* polymerase (Fig. 2). Thus, natural transformation may provide a simple measure of the relative fidelity of procedures for amplifying DNA with PCR.

Characterization of mutations within *pobR*. Twenty-five preparations of *pobR* DNA amplified separately with *Taq* polymerase were used to prepare 4-hydroxybenzoate-resistant transformants from strain ADP230. No more than 10 colonies were selected from each transformation to reduce the chance of picking identical mutants caused by accumulation of specific mutations during PCR. Wild-type DNA corresponding to the Δ*pcaBDK1* deletion was restored to each of the 247 strains that were analyzed. Of these, 243 demonstrated the ability to grow with protocatechuate and therefore appeared to be blocked in *pob* expression. Interestingly, a range of mutant phenotypes were observed when growth was tested on 4-hydroxybenzoate as the carbon source: null (no growth at all), heat sensitive (little or no growth at 37°C), cold sensitive (little or no growth at 22°C), and leaky (slow growth at both 22 and 37°C).

The 243 mutations were mapped by marker rescue with nested deletion fragments of the wild-type *pobA-pobR* region, as described previously (1, 4), and most of the mutations were shown to be distributed fairly evenly within *pobR* (Fig. 3). Four of the mutations were near the start of *pobA* and therefore could have been generated from the amplified *pobR* fragment (Fig. 1A). Of the 243 strains, a minimum of 143 could be regarded as having acquired independent mutations in *pobR* because of their origin (PCR batch), location (Fig. 3), or phenotype (null, leaky, or temperature sensitive). In addition, eight strains contained mutations in the *pobA-pobR* intergenic region.

Of the 143 strains with a demonstrated independent mutation within *pobR*, only 96 exhibited a null phenotype. Of the remaining strains, 17 were leaky, 16 were heat sensitive, and 14 were cold sensitive. Thus PCR mutagenesis provided a range of mutants, many of which (in this case, 33%) are likely partially defective because of minor changes in PobR. Such mutations provide convenient probes for analysis of residues important in protein structure and function. In contrast, repeated selection for spontaneous mutants blocked in *pobA* or *pobR* almost exclusively yielded strains with a completely null phenotype.

The nucleotide sequences of *pobR* and the upstream *pobA-pobR* intergenic region were determined in 83 independent *pobR* mutants and in 6 strains mutated in the intergenic region. The types of mutations are summarized in Table 1. Almost all of the mutations were nucleotide substitutions: the only other kind of mutations were frameshifts due to a deletion (two strains) or insertion of a nucleotide (one strain [Table 1]). The vast majority (76 strains) carried a single-base change. Multiple

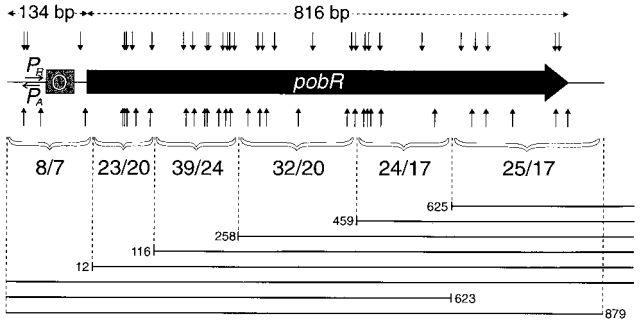

FIG. 3. Distribution of 151 independent PCR-generated mutations throughout the 816-bp *pobR* and the 134-bp *pobA-pobR* intergenic region. The mutations were selected in strain ADP230 (Δ*pcaBDK1*). After this mutation was replaced with wild-type DNA, the PCR-generated *pob* mutation was the sole barrier to growth with 4-hydroxybenzoate. The mutations were mapped by transforming the mutant cells with nested deletion fragments of wild-type *pobR*. *Acinetobacter* DNA present in these fragments is indicated by the horizontal lines below *pobR*; adjacent numbers refer to the nucleotide sequence that marks the beginning of the deletion in the *pobR* nucleotide sequence. Vertical dashed lines ascend from this position and connect to the ends of brackets marking separate DNA segments where mutations were mapped. Numerals directly below the brackets give additional information about the distribution of the mutations: to the left of each slash is the minimum number of selected mutations in the region marked by the bracket, and to the right of each slash is the number of independent PCR amplifications that give rise to the mutations. Sequencing of 89 of the mutations revealed their precise location within the segment in which they had been mapped. The locations of 62 of these mutations (all single-nucleotide substitutions causing amino acid substitutions) are indicated by the vertical upward and downward arrows. Promoter and operator regions are indicated as in Fig. 1B.

substitutions in *pobR* were found in 10 strains, one of which carried as many as four mutations. None of the mutations was caused by IS*1236*, the predominant cause of spontaneous mutation in *Acinetobacter pobR* (10). With the exception of transversion of G to C, all possible transitions and transversions were observed (Table 1). There was a strong bias for T-to-C transitions as well as for A-to-T and T-to-A transversions. It can be concluded that transformation-facilitated PCR mutagenesis of *Acinetobacter pobR* yields a wide range of point mutations that would not be observed within any manageable sample of spontaneous mutant strains.

Mutations in the *pobA-pobR* intergenic region. The *pobA-pobR* intergenic region is exceptional in that promoters for the two divergently transcribed genes are superimposed in a 33-bp DNA segment (3, 5) (Fig. 4). The necessity of this region for *pob* expression is highlighted by the fact that four of six sequenced mutations in the intergenic region are positioned so as to affect the promoters; these mutations all confer a null phenotype (i.e., no growth on 4-hydroxybenzoate at either 22 or 37°C. The remaining two mutations do allow growth at 37°C but prevent growth at 22°C and are in positions that are likely to affect translation of the *pobR* open reading frame (Fig. 4): *pob-1469* alters the ribosomal binding site in a manner that would reduce its capacity to bind to the complementary 3′ region in *Acinetobacter* 16S rRNA (5′-GAUCACCUCCUU-3′ [40]), and *pob-1475* is a T-to-G transversion 2 bp upstream of the translational start of *pobR*. The latter finding is in agreement with the observed low frequency of G's in the area between the ribosomal binding site and the translational initiation codon (11).

PCR-generated suppressor mutations. As suggested above, subtleties of molecular interaction can be revealed by conditional mutations. Such interactions can also be revealed by second-site suppressor mutations that can be produced by PCR amplification. The potential power of the technique was illustrated by suppressing the heat-sensitive Δ*pcaG1102* mutation, a 30-bp deletion that prevents formation of a functional protocatechuate 3,4-dioxygenase at 30°C. This deletion of residues 78 through 87 in the primary sequence of the protein's α subunit, encoded by the *pcaG* gene, removes most of a loop involved in interactions with the β subunit, encoded by the *pcaH* gene (26, 38). PCR amplification with *Taq* polymerase across the Δ*pcaG1102* mutation created DNA that gave rise to transformants of the parental deletion strain that had regained the ability to use protocatechuate at 30°C. The *pcaG* and *-H* genes from six such transformants, independently derived with six different PCRs, were sequenced, and a point mutation creating an amino acid substitution was found in *pcaG* in each case. In two cases, the intragenic suppressor mutation was Leu_{39} to Phe_{39}, created by changing TTG to TTT, and in the remaining cases, Gln_{93} to His_{93} was created, three times by a CAA to CAC change and once by a CAA to CAT change. In order to confirm that the sequenced second-site mutation was the cause of suppression, PCR amplification across both mutations in the suppressed strains was used to create DNA that gave rise to over 1,000 times more transformants of the parental strain than in the initial selection.

Selection of codons after random mutagenesis. In order to study structure-function relationships, it is useful to know what nucleotide substitutions are acceptable at a specified codon. In principle, selection might operate either at the level of nucleic

TABLE 1. Nature of PCR-induced *pobR* mutations determined through sequence analysis of the complete PobR-encoding region in 89 PobR mutants

Nature of mutation	No. of mutants
General	
Total no. of mutants	89
Mutations in the *pobA-pobR* intergenic region	6
Mutations in *pobR*	83
Single-nucleotide substitutions in:	
pobA-pobR intergenic region	6
pobR	70
Amino acid change	62
Nonsense mutation	8
Frameshift in *pobR*	
Single-base deletion	2
Single-base insertion	1
Multiple point mutations in *pobR*	
Double	6
Triple	3
Quadruple	1
Specific[a]	
Total no. of single-nucleotide substitutions in *pobR* and *pobA-pobR* upstream region	76
Transitions	
A to G	5
G to A	1
T to C	15
C to T	3
Transversions	
A to T	14
T to A	18
C to G	1
G to C	0
A to C	5
C to A	4
T to G	7
G to T	3

[a] Specification of transitions and transversions in the 76 *pobR* mutants carrying a single-nucleotide substitution.

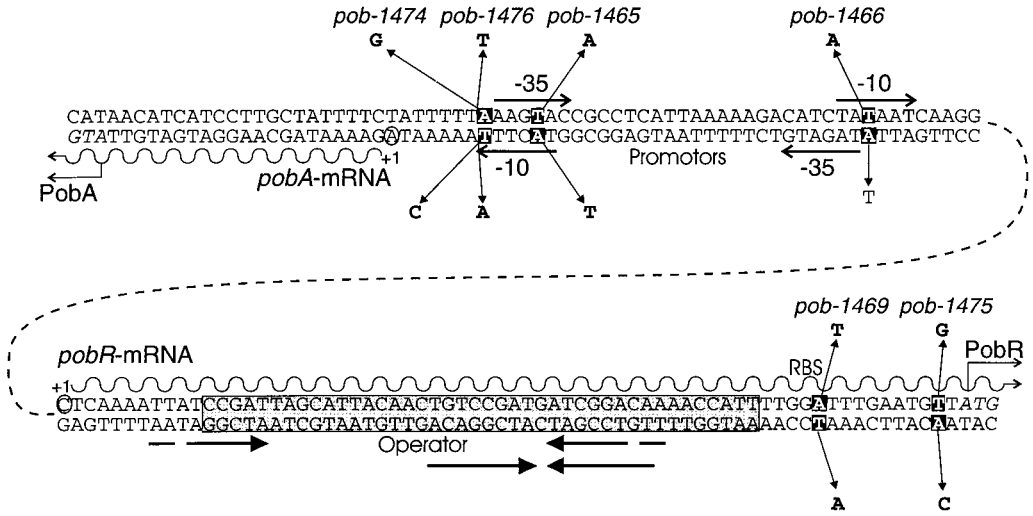

FIG. 4. Location of mutations in the *pobA-pobR* intergenic region. The contiguous nucleotide sequence between the translational starts of *pobA* and *pobR* is shown with promoter elements of both genes (5). Wavy lines mark the *pobA* and *pobR* mRNA with, +1 indicating their respective transcription initiation sites (nucleotides are circled). PobR binds to nucleotides enclosed in the shaded rectangle (operator); horizontal arrows indicated inverted repetitions of nucleotide sequence in this operator region. RBS indicates the presumed ribosome binding site on the *pobR* mRNA. Mutations *pob-1465*, *pob-1466*, *pob-1474*, and *pob-1476* are in positions that would allow them to block transcription. Mutations *pob-1469* and *pob-1475*, both cold sensitive, are in positions that would allow them to block translation of *pobR*.

acid (by demand for a particular codon) or at the level of protein (where functional constraints could limit the range of acceptable amino acid substitutions). These possibilities were explored by examination of phenotypic revertants prepared from two strains in which *pobR* translation had been terminated by stop codons. DNA containing the stop codons was amplified with *Taq* polymerase and used as a donor in crosses with the original mutants as recipients. To ensure that phenotypic revertants were the consequence of independent genetic events, 10 different transformations were performed with DNA that had been amplified separately. Recombinants that had acquired the wild-type phenotype were selected on solidified media containing 4-hydroxybenzoate. Typically, between 50 and 300 colonies would appear on plates in which 10^8 recipient cells had encountered PCR-amplified DNA, whereas no colonies appeared in the absence of DNA. The genetic basis for phenotypic reversion was determined by sequencing *pobR* DNA from one strain emerging from each of the 10 separate transformations.

One stop codon (TAG) replaced the wild-type TTG encoding a leucyl residue at position 154 in PobR. The amino acid residue is likely to be subject to functional constraints because it is conserved in *Acinetobacter* PcaU (9) and *Pseudomonas* PcaR (14, 31). As shown in Fig. 5A, all 10 selected phenotypic revertants were caused by restoration of the wild-type codon for leucine. Despite their likely occurrence, other mutations causing substitution of glutaminyl, glutamyl, lysyl, seryl, tryptophanyl, or tyrosyl residues at position 154 were not selected (Fig. 5A). This observation fortifies the interpretation that Leu_{154} serves a selected function in PobR and related transcriptional regulators.

The other stop codon (TGA) replaced the wild-type AGA encoding an arginine codon at position 40 in PobR (Fig. 5B). Use of AGA as a codon is quite infrequent in *Acinetobacter* structural genes (39), and the presence of the codon in transcriptional activators might be regarded as the result of selection for reduced expression of these genes by translational control (3). Alternatively, the unusual codons may simply have been accepted in transcriptional activators because there has been no demand for elevated expression of these genes. Evidence for selection of a basic amino acid residue at position 40 emerges from conservation of an arginyl residue in the aligned sequence of *Acinetobacter* PcaU and a lysyl residue in the aligned sequence of *Pseudomonas* PcaR. Such selective pres-

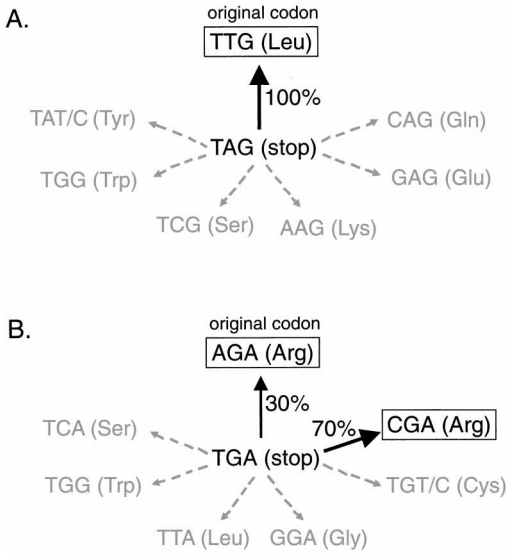

FIG. 5. Genotypic reversion of two PobR mutants in which *pobR* translation had been terminated by a stop codon: TTG to TAG (Leu_{154} to stop) (A) and AGA to TGA (Arg_{40} to stop) (B). Mutant PobR DNA amplified with *Taq* polymerase was used as donor DNA in transformation of the respective mutants, and phenotypic revertants were selected for growth on 4-hydroxybenzoate. Ten independent amplification and transformation experiments were performed for each mutant, and the reverted genotype was determined by sequence analysis of the *pobR* locus in 10 independent revertants. The original codons (top), mutant codons (middle), and the observed reverted codons (boxed) have been indicated in black (solid arrows), with the percentage of reversion among the 10 independently generated revertants given in boldface. Other potential codon reversions that could be obtained after a single-nucleotide substitution and that were not encountered have been marked in grey, with dashed arrows.

sures appear to operate because arginine was the only amino acid selected in the phenotypic revertants (Fig. 5B). An absence of selection for AGA is suggested by the fact that 70% of the phenotypic revertants substituted the arginine codon CGA for AGA in the wild-type strain (Fig. 5B).

DISCUSSION

Direct random PCR mutagenesis of chromosomal genes and rapid assessment of relative polymerase fidelity in PCR. Current protocols for random mutagenesis of bacterial genes through PCR all have to resort to the generation of plasmid-based libraries of partially mutated PCR fragments, which involves a cloning step in *E. coli* (2, 23, 34, 41). The results presented in this paper demonstrate that random point mutations can be introduced in specific chromosomal loci by coupling random PCR mutagenesis with natural transformation. The natural transformation system of *Acinetobacter* strain BD413 and derivatives allows for efficient chromosomal integration of polymerase errors through homologous recombination. Cloning of PCR fragments is therefore not required. The power of this procedure is illustrated by the high-level recovery and even distribution of single-nucleotide substitutions in and upstream of *pobR*, encoding the regulator of 4-hydroxybenzoate metabolism in *Acinetobacter.*

Mutation frequencies exceeded spontaneous mutation levels between 50- and 240-fold, depending on the polymerase used in amplification of wild-type chromosomal *pobR*. With saturating concentrations of donor DNA, these variations in mutation frequency should reflect the relative polymerase fidelity in PCR. The inferred 5-fold difference in fidelity between *Taq* polymerase and Pfu polymerase is lower than the 11- to 12-fold difference observed by Lundberg et al. (24), yet this may be explained by differences in PCR conditions and selected mutations between the two studies. The average error rate of *Taq* polymerase heavily depends on specific reaction conditions (2, 23, 34) and may range from 1 per 100,000 to as many as 1 per 2,000 nucleotides polymerized (6, 7, 41). The average error rate of *Taq* polymerase in our experiments, without optimization of PCR conditions, can be estimated to fall in the middle range.

Kohler et al. (20) have published a relatively easy method for analysis of polymerase fidelity based upon phage plaque counts after the inactivation of *lacI* during PCR, in vitro packaging of PCR products in phage λ, and subsequent infection of *E. coli*. We propose an easy alternative to this method that allows an overnight assessment of relative polymerase fidelity in PCR amplification: direct transformation of exponentially growing *Acinetobacter* strain ADP230 with amplified *pobR* DNA, followed by selection for resistance to 4-hydroxybenzoate. The frequency of resistant colonies is a direct relative measure of the PCR fidelity.

Targeted random mutagenesis of chromosomal genes. The aspect of targeted mutagenesis is best illustrated by comparison of the fraction of mutants affected in *pobA-pobR* among the total of mutants recovered from ADP230 (Δ*pcaBDK1*) that resist 4-hydroxybenzoate. While only one-fifth of the spontaneous mutants are affected in *pob* expression, the efficiency was virtually 100% after transformation with PCR-amplified *pobR* DNA. Similarly, two types of second-site suppressor mutations of the Δ*pcaG1102* deletion could be recovered after transformation with amplified Δ*pcaG1102* DNA. The natural frequency of occurrence of such mutations apparently is too low for them to be detectable among spontaneous mutants, which, in the case of Δ*pcaG1102* suppressors, were all located elsewhere on the chromosome. Similarly, reversion of the two *pobR* nonsense mutations was only observed in PCR recombinants: no spontaneous revertants were observed (in about 10^8 to 10^9 cells plated). Thus, the frequency of mutations in a specific chromosomal locus can be lifted above detection levels by combining PCR mutagenesis with efficient natural transformation.

Altered spectrum of chromosomal mutations. Analysis of the frequency of mutations in specific loci also touches upon another important aspect, which is that the spectrum of recovered PCR-induced mutations in *pobR* differs significantly from the spectrum of spontaneous *pobR* mutations. The latter are mostly caused by IS*1236* insertions (10). Among the total of spontaneous mutations recovered from strain ADP230, insertions and deletions of various kinds are detected with high frequency, as exemplified by mutations in *pcaH* and *pcaG* (8). Analysis of such mutations has suggested mechanisms that led to their occurrence (8, 12, 27). However, in selection of mutations primarily for structure-function analysis of proteins, major chromosomal rearrangements are inconvenient.

As shown in this paper, large insertions (e.g., IS*1236*) and deletions were not detectable among PCR-induced mutations recovered from the chromosome. Instead, the vast majority of PCR-induced mutations form a broad spectrum of nonpolar single-nucleotide substitutions relatively evenly distributed in *pobR*. Analysis of the nature of each mutation individually falls outside the scope of this paper, but the summation of mutations shows that T-to-C transitions as well as A-to-T and T-to-A transversions occurred at relatively high frequency. A strong bias of *Taq* polymerase for T-to-C transitions has been documented (36) and apparently can be largely overcome by altering the amplification reaction conditions (2). The observed high frequency of A-to-T transversions has not been described previously and may therefore primarily be the result of phenotypic constraints in selection of the recovered *pobR* mutations.

The nature of mutations recovered after PCR-induced mutagenesis allows for direct in vivo analysis, in a chromosomal (single-copy) context, of their effects on structure and activity of *Acinetobacter* PobR. This is especially effective for identification of small modifications to the wild-type protein that cause a leaky or conditional phenotype, as is observed for 33% of the PCR-induced *pobR* mutations. Clearly, introduction of PCR mutations through transformation provides a way to gently probe the residues essential to the structure and activity of *Acinetobacter* PobR. Significantly, leaky or conditional mutations are rarely found among spontaneous PobR mutations, another demonstration of the altered mutation spectrum.

Suppression mutations and codon randomization. PCR mutagenesis produced two different second-site mutations suppressing the 30-bp deletion Δ*pcaG1102*. The exact nature of this suppression at the level of the protein remains unclear. However, the fact that both suppressors are near the original deletion highlights the potential for fluidity in this region of the protein (26, 37, 38) and provides targets for future site-directed mutagenesis studies.

The experiments in which PobR nonsense mutations were restored to functional alleles indicate that the wild-type codons (Arg_{40} and Leu_{154}) are both rather essential to the activity of PobR in vivo. Similar experiments can essentially be performed with any type of point mutation that has a clear phenotype, and such mutations are numerous among strains recoverable after PCR-induced mutagenesis. Because this type of experiment is not limited to nonsense mutations, the codon reversion strategy proposed could yield a widely applicable alternative to codon randomization experiments. Transformation-facilitated PCR mutagenesis can thus partially serve as an

easy alternative to the use of vast amounts of tRNA suppressor strains (19).

In conclusion, we have verified an extremely easy and efficient procedure for targeted random mutagenesis of chromosomal genes employing natural transformation. A rapid assay for assessment of relative polymerase fidelity in PCR amplification follows from the direct selection of 4-hydroxybenzoate resistance in *Acinetobacter* strain ADP230. Provided a selection for mutations exists, analysis of a wide range of chromosomal point mutations should be applicable to other genes in *Acinetobacter* and in other natural transformation systems. Second-site suppression mutations as well as specifically randomized codons should facilitate both protein structure-function studies and analysis of the directed evolution of proteins.

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