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When Coupled to Natural Transformation in *Acinetobacter* sp. Strain ADP1, PCR Mutagenesis Is Made Less Random by Mismatch Repair†

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Random PCR mutagenesis is a powerful tool for structure-function analysis of targeted proteins, especially when coupled with DNA integration through natural transformation followed by selection for loss of function. The technique has been applied successfully to structure-function analysis of transcriptional regulators, enzymes, and transporters in *Acinetobacter* sp. strain ADP1. However, the mismatch repair system prevents the full spectrum of nucleotide substitutions that may be selected at the level of protein function from being recovered. This barrier may be overcome by introducing PCR-mutagenized genes into strains in which the corresponding genes have been deleted.

Random PCR mutagenesis provides an array of mutations, many of which cause amino acid substitutions that may alter the functional properties of proteins. When coupled to natural transformation in *Acinetobacter* sp. strain ADP1, the procedure can be streamlined, as PCR amplicons of targeted genes are provided directly to the host and integrated into the chromosome by homologous recombination (15). When followed by a suitable screen or selection, the process can provide an overview of the amino acid side chains that contribute to function. A merit of the procedure is its objectivity because mutations are identified on the basis of phenotypic variation. No presumptions are brought to bear on the identification of targets for mutagenesis, so amino acid contributions that might not have been predicted can be characterized (7, 10, 24). Nevertheless, the procedure must be regarded with caution because not all nucleotide substitutions are made with equal probability during PCR amplification (34). Furthermore, as described here, mismatch repair (MMR) also contributes to making the process less than fully random because it reduces the frequencies of transition mutations that are introduced into the chromosome.

Transformation-facilitated random PCR mutagenesis has been successfully applied to proteins involved in the catabolism and regulation of phytochemicals in the nutritionally versatile *Acinetobacter* sp. strain ADP1. These investigations have given valuable insight into structure-function relationships in these proteins (7, 15, 16, 17, 24, 29). However, when we applied the random PCR mutagenesis approach to *pcaK*, a gene encoding a protocatechuate transporter belonging to the ubiquitous major facilitator superfamily (28), evidence of bias in the mutant collection was observed. A library of 117 *pcaK* mutants was analyzed, and in 20 cases, identical base pair substitutions were recovered in independently derived mutants (A. Buchan and L. N. Ornston, unpublished data). While saturation was being approached with respect to the field of *pcaK* variants that could be recovered using this technique, absent from the collection were several anticipated mutations, including those abolishing conserved charged residues in transmembrane-spanning regions. Furthermore, the nucleotide substitutions were heavily biased towards transversions (71%) relative to transitions (29%). This pattern contrasts the distribution of nucleotide substitutions recovered from random PCR mutagenesis approaches coupled with cloning and expression of a target gene in *Escherichia coli* (Fig. 1). As generation of PcaK mutants in *Acinetobacter* sp. strain ADP1 depends upon replacement of the wild-type *pcaK* allele with mutated variants via homologous recombination, a transformation-associated bias caused by MMR was considered.

Prior studies of MMR systems of divergent bacterial lineages provide a framework for how cellular machinery might block integration of transition-containing PCR products in *Acinetobacter*. MMR plays a well-recognized role in repair of replication-based errors, but it is also active in preventing recombination between divergent sequences (31, 32, 38, 39). Heteroduplex DNA containing even single-nucleotide mismatches formed during homologous recombination can be targets of MMR-mediated heteroduplex rejection, also known as antirecombination (5, 23). MutS, the mismatch binding protein and an essential component of MMR, preferentially binds mismatches that arise from transition mutations over those arising from transversion substitutions (3), thereby affecting the frequency with which mutant alleles are integrated into the chromosome during recombination (11). Furthermore, recent studies of *E. coli* have demonstrated that MMR-mediated sequence-specific biases in genetic engineering approaches employing bacteriophage λ are relieved with MutS-defective cells (6). Therefore, an interpretation of the conflicting substitution profiles presented in Fig. 1 is that the *Acinetobacter* MMR system reduces the integration efficiency of transition mutation-bearing PCR

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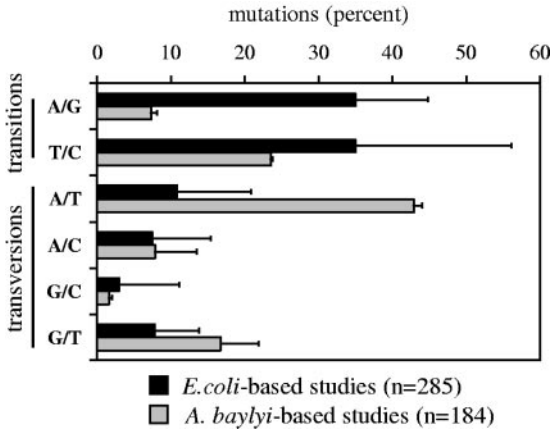

FIG. 1. Distribution of single-nucleotide substitutions generated by a general random PCR mutagenesis approach (41). Natural transformation through which heteroduplex DNA was formed (25, 26) was used to obtain *Acinetobacter* mutants used in this analysis (15 [$n = 76$]; A. Buchan and L. N. Ornston, unpublished data [$n = 108$]). Mutants derived from *E. coli*-based approaches in which heteroduplex DNA is not formed were obtained from the following references: references 18 ($n = 48$), 30 ($n = 14$), 1 ($n = 10$), 36 ($n = 35$), 2 ($n = 8$), 33 ($n = 9$), 35 ($n = 6$), 42 ($n = 8$), 9 ($n = 5$), 19 ($n = 18$), 37 ($n = 11$), 40 ($n = 13$), 27 ($n = 19$), 4 ($n = 5$), and 20 ($n = 76$). If nucleotide substitutions are not explicitly described in the references, they were deduced from protein substitutions, and ambiguous substitutions were omitted. Error bars represent variations in frequencies among studies conducted with the two organisms. *A. baylyi*, *Acinetobacter baylyi*.

products during the transformation step. To test this hypothesis, PCR mutagenesis of *pcaK* was conducted with strains containing or lacking functional MutS, and the resulting nucleotide substitutions in the mutant genes were compared.

Acinetobacter strain ADP8502 contains the Δ*vanK7602* and Δ*pcaBD* mutations that facilitate selection of PcaK-deficient strains on medium containing protocatechuate and a nonselecting growth substrate (7). The basis of the selection is twofold. First, as both VanK and PcaK transport protocatechuate (8), selection for strains defective in PcaK activity is carried out in a Δ*vanK* background. Second, PcaB-deficient strains that take up and metabolize protocatechuate accumulate a toxic metabolite that inhibits growth on the selective medium (12). Inactivation of MutS in strain ADP8504 was achieved by an Ω(SpR/StR) insertion by the following procedure: strain ADP8502 was transformed with the linearized plasmid pZR7008 (38) followed by selection for resistance to streptomycin and spectinomycin at respective concentrations of 10 and 40 μg/ml. PCR mutagenesis was performed using crude cell lysates of *Acinetobacter* sp. strain ADP1 with the primers PK1 and PK2 (7) in 100-μl volumes. Twenty-five microliters from each reaction mixture was added in parallel to 500-μl competent cultures of ADP8502 and ADP8504. Following 3 h of incubation at 37°C with shaking, the transformation reaction mixtures were plated on minimal medium (31) containing 5 mM fumarate and 1 mM protocatechuate and incubated in the dark at 22°C for 28 h. The Δ*pcaBD* deletion was corrected by transformation of purified strains with a plasmid carrying wild-type *pcaBD* (pZR8550). The *pcaK* genes of mutant strains were sequenced in their entirety by using PK1, PK2, and an internal primer, PK3 (5′ CCGATTTCTAACAGGTATCGG ATTG 3′). *pcaK* was sequenced from representative mutants derived from ADP8502 ($n = 13$) and ADP8504 ($n = 18$) transformed with *Taq*-amplified *pcaK* DNA. Substitutions are presented relative to the *pcaK* coding strand and as "nucleotide/nucleotide" (e.g., T/C). The arrangement of the nucleotide designations does not necessarily reflect the original substitution (i.e., T/C represents either a T-to-C or C-to-T substitution).

Comparison of PCR-generated mutations in strains containing and lacking functional MutS. Consistent with the mutator phenotype typical of MutS-deficient strains (5), the frequency of spontaneous mutations giving rise to protocatechuate-resistant colonies was sevenfold higher in strain ADP8504 than that in strain ADP8502. Transformation of ADP8504 with *Taq*-amplified *pcaK* DNA resulted in 30-fold more protocatechuate-resistant colonies than did that of the same strain receiving no PCR product (see Fig. S1 in the supplemental material). This is similar to the 24-fold increase seen when amplified products are provided to the wild-type *mutS* strain ADP8502 and suggests that more than 95% of the protocatechuate-resistant colonies derived from either strain transformed with *pcaK* PCR products possess *Taq*-introduced mutations.

The spectrum of PCR-generated mutations identified in the MutS-deficient strain (ADP8504) receiving *pcaK* PCR products was distinct from that found with the MutS-positive strain (ADP8502). Transitions accounted for 84% of all *pcaK* nucleotide substitutions derived from the MutS-deficient strain compared to 42% of substitutions obtained from ADP8502 provided with the same PCR product pools (see Table S2 in the supplemental material). The substitution profiles for the 12 *pcaK* variants obtained in the functional MutS background parallel those found in a previous study of PCR-generated mutations in *pcaK* (A. Buchan and L. N. Ornston, unpublished data) and were folded into the larger data set for comparisons. This substitution pattern was also remarkably consistent with that of another large mutant library generated in *Acinetobacter* sp. strain ADP1 (PobR) (see Table S1 in the supplemental material) (15). Relative to substitutions recovered in functional MutS strains, the frequencies of both A/G and T/C transitions were elevated about three- and twofold, respectively, in the MutS-deficient strain. A second feature characteristic of the PcaK mutants derived from the MutS-deficient strain was that most (73%) contained multiple (ranging from two to five) mutations within *pcaK* (see Table S2 in the supplemental material). In contrast, only 15% of the mutants derived from the functional MutS strain receiving the same PCR product pools had more than one mutation in *pcaK*, a finding consistent with previous studies of PcaK and PobR (see Table S1 in the supplemental material). However, significantly higher frequencies of multiple mutations at a target locus have been reported using this approach (24). As MMR systems are subject to saturation (13, 21), the prevalence of multiple mutations in a target gene is likely a reflection of the capacity of the MMR system and the composition of the PCR pool provided as donor DNA.

Circumventing the transformation-associated bias. Application of the transformation-facilitated random PCR mutagenesis approach to a heterologous gene yielded a different pattern of nucleotide substitutions. Kok et al. (15) designed a docking site in the *Acinetobacter* sp. strain ADP1 chromosome that would allow integration and expression of foreign genes

and PCR-mutagenized variants of that gene. In this system, the recipient strain does not have DNA corresponding to the gene targeted for PCR mutagenesis. Of the 25 nucleotide substitutions identified in 17 mutants, 92% were transitions. This pattern is analogous to that of *E. coli*-based libraries (see Table S1 in the supplemental material) and consistent with our interpretation of the role of MMR. If the gene targeted for PCR mutagenesis is not present in the chromosome of the *Acinetobacter* recipient strain, heteroduplexes are avoided and the MMR-mediated biases associated with the transformation-facilitated random PCR mutagenesis approach are circumvented. Therefore, through chromosomal deletion of the gene of interest and the use of a counterselectable marker, such as *sacB* (14, 22), to facilitate integration of mutagenized variants, it is likely that a different spectrum would be recovered. It would be beneficial to take this approach in the presence of a functional MutS because, as noted above, deficiencies in MutS give rise to a high frequency of strains with multiple mutations in the target gene.

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