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Phenotypic Expression of PCR-Generated Random Mutations in a *Pseudomonas putida* Gene after Its Introduction into an *Acinetobacter* Chromosome by Natural Transformation†

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Localized sets of random point mutations generated by PCR amplification can be transferred efficiently to the chromosome of *Acinetobacter* ADP1 (also known as strain BD413) by natural transformation. The technique does not require cloning of PCR fragments in plasmids: PCR-amplified DNA fragments are internalized by cells and directly incorporated into their genomes by homologous recombination. Previously such procedures for random mutagenesis could be applied only to *Acinetobacter* genes affording the selection of mutant phenotypes. Here we describe the construction of a vector and recipient that allow for mutagenesis, recovery, and expression of heterologous genes that may lack a positive selection. The plasmid carries an *Acinetobacter* chromosomal segment interrupted by a multiple cloning site next to a kanamycin resistance marker. The insertion of heterologous DNA into the multiple cloning site prepares the insert as a target for PCR mutagenesis. PCR amplifies the kanamycin resistance marker and a flanking region of *Acinetobacter* DNA along with the insert of heterologous DNA. Nucleotide sequence identity between the flanking regions and corresponding chromosomal segments in an engineered *Acinetobacter* recipient allows homologous recombination of the PCR-amplified DNA fragments into a specific chromosomal docking site from which they can be expressed. The recipient strain contains only a portion of the kanamycin resistance gene, so donor DNA containing both this gene and the mutagenized insert can be selected by demanding growth of recombinants in the presence of kanamycin. The effectiveness of the technique was demonstrated with the relatively GC-rich *Pseudomonas putida xylE* gene. After only one round of PCR amplification (35 cycles), donor DNA produced transformants of which up to 30% carried a defective *xylE* gene after growth at 37°C. Of recombinant clones that failed to express *xylE* at 37°C, about 10% expressed the gene when grown at 22°C. The techniques described here could be adapted to prepare colonies with an altered function in any gene for which either a selection or a suitable phenotypic screen exists.

The analysis of how structure influences the function of a protein benefits from the availability of a spectrum of point mutations in the encoding gene. The randomness of nucleotide substitutions by thermostable polymerases in the PCR makes it a good candidate for the generation of such mutations (1, 15, 22, 23, 25). The investigation of how random mutations may change the properties of a protein benefits from a biological system in which their individual phenotypes may be either selected or screened in vivo, preferably expressed from a chromosomal background.

Recently it has been shown that PCR-generated mutations can be targeted to chromosomal *Acinetobacter* genes by the direct coupling of mutagenesis during PCR amplification to the uptake of the amplified DNA segments by natural transformation (10, 11). This procedure allows for the easy recovery of strains carrying nonpolar single nucleotide substitutions in chromosomal genes, provided there is a selection for the mutant phenotype (10, 11). Thus, a vast number of independently generated mutant alleles, many with conditional phenotypes, were recovered from the chromosomal *pobR* gene encoding the regulator of 4-hydroxybenzoate degradation in *Acinetobacter* (4, 5). *Acinetobacter* forms an ideal recipient for the chromosomal integration of PCR-generated alleles, as the natural transformation system of the organism (6–8, 18, 19) is highly efficient and accepts linear DNA fragments that have been amplified by PCR. Moreover, unlike many other transformable organisms, *Acinetobacter* displays a constitutive elevation of the level of recombination (RecA) activity that does not require specific induction upon induction of competence for natural transformation (21).

A limit to the mutagenesis system described above is that it requires a selection for strains containing mutant alleles. In addition, it can only be applied to genes from naturally transformable organisms. To overcome these limitations, we have constructed a specific *Acinetobacter* recipient (ADP1200) and a cloning vector (pZR80) that together allow for the easy chromosomal recovery of mutant alleles of virtually any gene of either homologous or heterologous origin (Fig. 1). The cloned gene is expressed from a constitutive promoter and is amplified together with a functional kanamycin marker by PCR. The PCR fragments are used directly as donor DNA in the transformation of the *Acinetobacter* recipient strain, leading to the integration of the PCR-amplified DNA into a chromosomal docking site formed by the *Acinetobacter lipBA* operon (13, 14). Selection for kanamycin resistance results in a population of *Acinetobacter* strains, each carrying a PCR-generated copy of the cloned gene. The procedure yields only cells that have incorporated the heterologous gene into their chromosomes. This allows for ready screening of colonies in which the gene product has an altered function. In the example given here, up to 30% of the strains retrieved after kanamycin selection expressed a defective mutant allele of the cloned heterologous *xylE* gene from *Pseudomonas putida*.

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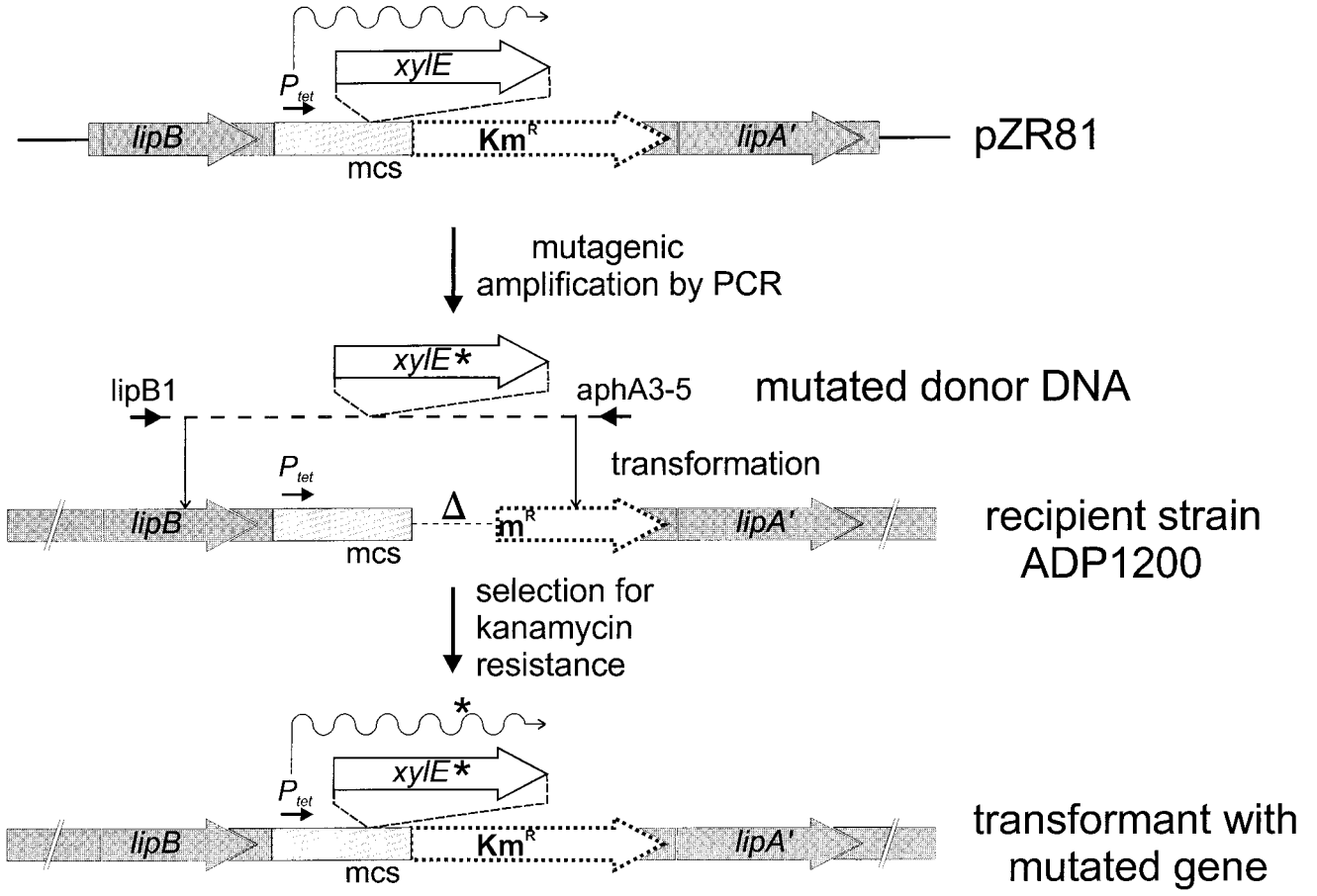

FIG. 1. Random PCR mutagenesis of a heterologous gene (exemplified by *P. putida xylE*) and recovery of mutated genes for expression from a targeted site in the *Acinetobacter* chromosome. The plasmid pZR81 was prepared by the insertion of *xylE* into the multiple cloning site (mcs) of expression vector pZR80, downstream from the constitutive *tet* promoter (P_{tet}), and flanked by sequences homologous to the chromosome of recipient strain ADP1200. Strain ADP1200 lacks a functional *aphA3* gene encoding kanamycin resistance. PCR was used to amplify *xylE* together with portions of *lipB* and *aphA3*, the latter encoding resistance to kanamycin. PCR DNA that may carry a PCR-generated mutation (*) was used to transform ADP1200 to kanamycin resistance. A significant fraction of the kanamycin-resistant recombinants contained a mutant allele of the *P. putida xylE* docked into the chromosome of ADP1200. Thus, the procedure provides a way to produce and express single-copy mutant alleles of genes that lack a selectable mutant phenotype.

MATERIALS AND METHODS

Construction of pZR80 for PCR mutagenesis of DNA inserts. The basis of pZR80 is the ColE1 plasmid pALJA434 (12), which carries the *lipBA* operon of *Acinetobacter* ADP1 as a 3.0-kbp *Eco*RV-*Sal*I insert. Plasmid pZR80 was constructed in a triple ligation step from three fragments (Fig. 2).

(i) Fragment I. Plasmid pALJA434 contains a unique *Eco*RI site engineered near the start of *lipA*, in addition to a unique *Kpn*I site further downstream in *lipA*. pALJA434 was digested with *Eco*RI and *Kpn*I, and a 5.2-kbp fragment was isolated.

(ii) Fragment II. Vector pMTL21p (3) carries an asymmetric polylinker. The 185-bp *Eco*RI-*Eco*RV fragment of pBR322, carrying part of the *tet* gene with the constitutive *tet* promoter (P_{tet}), was cloned into pMTL21p, which had been digested with *Eco*RI and *Sma*I. The resulting plasmid was digested with *Eco*RI and *Sph*I, and a 247-bp fragment that contained most of the polylinker of pMTL21p downstream of P_{tet} was isolated (Fig. 2).

(iii) Fragment III. The kanamycin resistance gene (*aphA3*) of pZR80 was derived from pPJ1 (20). The *aphA3* gene was amplified by PCR, with pPJ1 as the template DNA and with primer aphA3-1 (5′-GAATGCATGCAACAGTGAA TTGGAGTTCG-3′), which anneals upstream of the *aphA3* promoter and carries an *Sph*I site (underlined) due to two base changes (double underlined), and aphA3-2 (5′-GCGGAGGTACCTCAGAAAAGATTAGATGTC-3′), which anneals downstream of *aphA3* and contains a *Kpn*I site due to two base changes. The resulting 963-bp PCR fragment was digested with *Sph*I and *Kpn*I to yield fragment III.

In a single forced-ligation step (Fig. 2), equimolar amounts of fragments I, II, and III were ligated to yield pZR80, with selection for resistance to ampicillin and kanamycin in transformed *Escherichia coli* DH5α. Vector pZR80 carries the constitutive promoter P_{tet} upstream of the polylinker of pMTL21p (with *Bam*HI, *Mlu*I, *Nco*I, *Bgl*II, *Xho*I, *Stu*I, and *Sph*I sites left as unique cloning sites) and the functional *aphA3* marker all cloned into the *Acinetobacter lipBA* operon (Fig. 2); the nucleotide sequence of part of pZR80 carrying P_{tet} and the polylinker (to the start of *aphA3*) is depicted in Fig. 3.

Construction of the recipient, *Acinetobacter* ADP1200. *Acinetobacter* ADP1200 was used as the recipient for transformation with PCR fragments amplified with pZR80-derived plasmids as template DNA (such as pZR81; Fig. 1). Strain ADP1200 carries an insert in the *lipBA* operon similar to the insert in pZR80, except that it lacks a 227-bp fragment carrying the *aphA3* promoter and the first part of the *aphA3* gene. A derivative of pZR80, designated pZR79, was used for the generation of ADP1200. Plasmid pZR79 was constructed essentially in the same way as that described for pZR80, except that an alternative to fragment III was created by using an internal *aphA3* primer, aphA3 (5′-GACGGCATGCC GGTATAAAGGGACCAC-3′), which carries an engineered *Sph*I site, in conjunction with primer aphA3-2. The resulting 736-bp PCR fragment was digested with *Sph*I and *Kpn*I to yield fragment IV, with a truncated *aphA3* gene. The ligation of fragment IV with the above-described fragments I and II yielded plasmid pZR79 (Fig. 2). After linearization with *Sca*I (which cuts in the vector part), pZR79 was used for the transformation of *Acinetobacter* ADP1. Strains were plated onto a nonselective medium to yield a few hundred colonies per plate, and colonies were transferred to lipase indicator plates with egg yolk and NaCl to be screened for loss of lipase activity, as described previously (9). Among 554 strains, seven failed to produce a turbid zone around the colony, indicating a loss of lipase activity. These strains did not express the ampicillin resistance marker of pZR79. Through PCR, one strain, designated ADP1200, was verified to contain the proper insert of fragments II and IV in the *lipBA* operon on the chromosome.

Construction of pZR81 and mutagenesis of *xylE*. Plasmid pZR81 was generated by cloning a 1.4-kbp *Nco*I-*Xho*I fragment from plasmid pUC18*Sfi*-HA, carrying the *xylE* gene from the no. 1 *meta* operon in the *P. putida* TOL plasmid pWW53 (17), between the unique *Nco*I and *Xho*I sites in the polylinker of

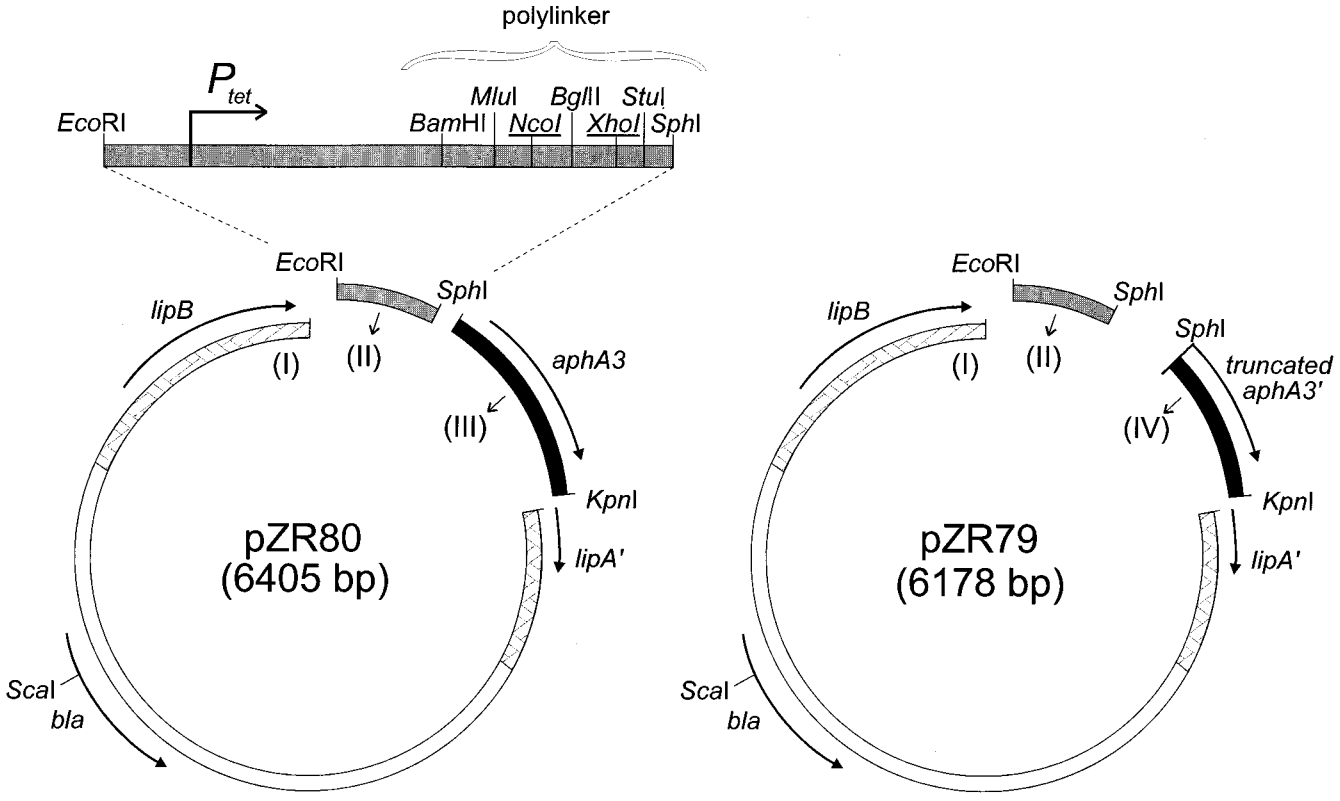

FIG. 2. One-step construction of plasmids pZR80 and pZR79 from three independent restriction fragments, numbered I (white), II (grey), and III (black) for pZR80 and I, II, and IV (black) for pZR79. The individual fragments have been marked, as have the three relevant restriction sites used for their cloning. *Acinetobacter* DNA present on the ColE1 vector fragment I is crosshatched. Fragment II has been enlarged at the top of the figure to show the presence of the constitutive P_{tet} and the unique cloning sites present downstream of this promoter in the small polylinker. Plasmid pZR79 was used to generate transformation recipient ADP1200 and carries a truncated *aphA3* gene (*aphA3′*; see the text).

pZR80 (Fig. 1 and 2), and selecting for resistance to ampicillin and kanamycin in transformed *E. coli* DH5α. The *xylE* gene does not carry its own promoter but is expressed from P_{tet} in pZR81 (Fig. 1). For the mutagenesis of the *P. putida xylE* gene, part of pZR81 was amplified with primers annealing in the *Acinetobacter lipB* gene, lipB1 (5′-TGCAGGGCTGTTCGGCTCAG-3′), and in *aphA3*, aphA3-5 (5′-GGCAATGTCATACCACTTGT-3′) (Fig. 1). The same primers were used to demonstrate that the inserts in recombinants failing to express *xylE* were the same size as the inserts in strains that expressed *xylE*.

```
EcoRI
GAATTCTCATGTTTGACAGCTTATCATCGATAAGCTTTAATGCGGTAGTTTATCACAGTT
             -35                      -10
            ---------- Ptet ------------>

AAATTGCTAACGCAGTCAGGCACCGTGTATGAAATCTAACAATGCGCTCATCGTCATCCT

CGGCACCGTCACCCTGGATGCTGTAGGCATAGGCTTGGTTATGCCGGTACTGCCGGGCCT

                                                    XhoI
             BamHI                  MluI   NcoI BglII    StuI
CTTGCGGGATGGGGATCCTCTAGAGTCGACGTCACGCGTCCATGGAGATCTCGAGGCCTG

    SphI
CAGGCATGCAACAGTGAATTGGAGTTCGTCTTGTTATAATTAGCTTCTTGGGGTATCTTT
                               -35                     -10
                            ------------ PaphA3 --------->

AAATACTGTAGAAAAGAGGAAGGAAATAATAAATGGCTAAAATGAGAATATCACCGGAAT
                                 Met----------AphA3--------->
```

FIG. 3. Nucleotide sequence of the region of pZR80 from *Eco*RI to the start of the functional kanamycin resistance gene *aphA3*. The fragment carries the promoter of the *tet* gene of pBR322 (P_{tet}) that is constitutive in *Acinetobacter* and part of the polylinker of pMTL21p (3) (doubly underlined). Unique restriction sites (in pZR80) that may be relevant for cloning have been marked above the polylinker. Relevant promoter elements have been marked and are shown in bold. The region that corresponds to the truncated *tet′* gene of pBR322 has been underlined. The start of the *aphA3* gene has been marked in italics, with the encoded initial methionine in AphA3 shown (Met).

Mutagenesis and screening differences in phenotypic expression of wild-type and mutant *xylE* genes after their introduction into *Acinetobacter* by transformation. *Taq* polymerase was used, as indicated by the supplier (Boehringer Mannheim), for the amplification of *xylE-aphA3* fragments for transformation. PCRs were carried out with 200 nM concentrations each of primers lipB1 and aphA3-5, 200 μM concentrations of each deoxynucleoside triphosphate, between 5 and 10 ng of template DNA (pZR81), and 0.5 U of polymerase in a final volume of 50 μl. The thermocycle protocol consisted of a total of 35 cycles, with a denaturation step at 94°C, primer annealing at 58°C, and elongation at 72°C. The DNA fragments produced by PCR were isolated from an agarose gel and directly used to transform ADP1200, according to the standard procedure for the natural transformation of ADP1-derived strains (10), followed by selection for recombinants on a mineral medium with 10 mM succinate supplemented with 30 μg of kanamycin per ml. Ten preparations of *xylE-aph3A* DNA were amplified separately with *Taq* polymerase and used to prepare kanamycin-resistant transformants from strain ADP1200. No more than 10 kanamycin-resistant recombinants were selected after each transformation to reduce the chance of picking identical mutants. Selected strains were subsequently screened for the expression of either functional or defective XylE by spraying colonies formed overnight with a 100 μM catechol solution.

Sequence analysis of mutations. The primers lipB1 and aphA3-5 (Fig. 1) were used at concentrations of 40 nM to amplify mutant *xylE* DNA from the chromosome with *Taq* polymerase. 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). Further sequencing procedures were described previously (10).

Nucleotide sequence accession number. The nucleotide sequence of the *xylE* insert in pZR81 has been deposited with GenBank under accession no. AF102891.

RESULTS

Integration of wild-type and mutated *P. putida xylE* genes into the *Acinetobacter* chromosomal docking site. After the transformation of ADP1200 with pZR81 DNA that had replicated in vivo, all 270 tested kanamycin-resistant recombinants expressed the GC-rich *P. putida xylE* gene, as evidenced by α-hydroxymuconic semialdehyde formation. In contrast, only 70% (443 of 629 colonies tested) of recombinant colonies emerging after transformation with PCR-amplified DNA produced a functional enzyme, as judged by the formation of yellow color from catechol after growth of the cells at 37°C. Of the colonies that failed to express functional XylE at 37°C, 10% formed active XylE during growth at 22°C. It therefore is apparent that the *xylE* gene in these recombinant strains contained a conditional mutation allowing the formation of a functional gene product at the lower temperature.

The presence of a DNA insert with predicted size and location in the recombinant strains was demonstrated with primers lipB1 and aphA3-5 (Fig. 1). Ten strains that failed to express *xylE* produced a DNA fragment of 3.1 kb; strains lacking the *xylE* insert formed a DNA fragment of 0.6 kb (data not shown). Therefore, it can be concluded that the failure of these strains to express *xylE* is due to a defective *xylE* gene rather than the absence of the gene in strains that had acquired the kanamycin resistance marker.

Nucleotide sequences of mutant *xylE* genes. In order to demonstrate the ease of phenotypic observation of PCR-generated mutations introduced into *Acinetobacter* by transformation, highly mutagenized *xylE* was used as a donor; about 30% of the resulting transformants exhibited defects in XylE (Fig. 1). As might be expected, a sample of *xylE* from strains with null phenotypes revealed a number of genes with multiple mutations (Table 1). Therefore, sequencing focused upon genes exhibiting either a heat-sensitive phenotype or a leaky phenotype, because it seemed likely that XylE from such organisms had undergone relatively subtle mutations impairing but not destroying enzyme activity. Most of these strains contained *xylE* with a single nucleotide substitution (Table 1).

TABLE 1. Genotypes and phenotypes of *Acinetobacter* mutant strains

Strain	Mutant gene(s)	*xylE* substitutions[a]	XylE substitutions	Phenotype
ADP1203	None	None	None	Wild type
ADP1204	*xylE1204*	C559T	R187C	Heat-sensitive
ADP1205	*xylE1205*	A371G	E124G	Heat-sensitive
ADP1206	*xylE1206*	T208C	F70L	Heat-sensitive
	xylE1306	G853A	D285N	
ADP1207	*xylE1207*	T893C	L298P	Heat-sensitive
ADP1208	*xylE1208*	A7G	K3E	Heat-sensitive
ADP1210	*xylE1210*	T502C	F168L	Heat-sensitive
ADP1212	*xylE1212*	T469A	Y157N	Null
ADP1213	*xylE1213*	C413T	A138V	Heat-sensitive
ADP1214	*xylE1214*	C12T (GCC → GCT)	Silent	Heat-sensitive
	xylE1314	A245G	E82G	
ADP1215	*xylE1215*	A21G (CGA → CGG)	Silent	Leaky
ADP1216	*xylE1216*	G168A (GTG → GTA)	Silent	Heat-sensitive
	xylE1316	G782A	G261D	
ADP1217	*xylE1217*	A703G	T244A	Heat-sensitive
ADP1219	*xylE1219*	C880T	H294Y	Heat-sensitive
ADP1220	*xylE1220*	A185G	E62P	Null
	xylE1320	T575C	L192P	
ADP1221	*xylE1221*	T502C	F168L	Null
	xylE1321	G688A	D230N	
ADP1222	*xylE1222*	G39C (CTG → CTC)	Silent	Null
	xylE1322	T542C	L181P	
ADP1227	*xylE1227*	T14C	V5A	Null
	xylE1327	A730G	T244A	
	xylE1328	A884G	D295G	

[a] Silent codon changes are shown in parentheses.

DISCUSSION

Direct phenotypic identification of genetic defects generated by PCR. A benefit offered by *Acinetobacter* natural transformation is that the phenotypic consequence of a mutation can be expressed in a recombinant colony shortly after the exposure of recipient cells to the modified DNA. In the present system, the mutated genes are expressed in a single copy from the recombinant chromosome, so that recessive alleles are unlikely to be masked by their dominant counterparts as might happen with genes expressed from multicopy plasmids. The *tet* promoter used here allows the constitutive expression of the cloned gene, so that defective genes can be identified in cells grown without an inducer. All that is required for the identification of phenotypic variants of the amplified gene is a simple screening procedure that monitors the activity of the gene product.

Comparison of amino acid substitutions causing XylE defects with amino acids that have been conserved during evolutionary divergence of XylE proteins. In order to assess the significance of PCR-generated amino acid substitutions in XylE, they were compared with residues that have been conserved in divergent proteins from three distant bacterial genera (Fig. 4). As a rough estimate, it might be assumed that highly conserved amino acid residues would be relatively sensitive to mutations causing defects that would be evident in the phenotypic screen. On the other hand, amino acid substitutions at loci where divergence had been accommodated during evolution might be expected to escape detection. To some extent these expectations were fulfilled. Thus, the substitutions E124G and G261D cause radical chemical changes in conserved residues and result in defective enzymes. Perhaps more noteworthy is the fact that the mutant enzymes are only partially defective, because they function at room temperature. Since the different cell lines are unlikely to have encountered elevated temperatures frequently during their evolution, the se-

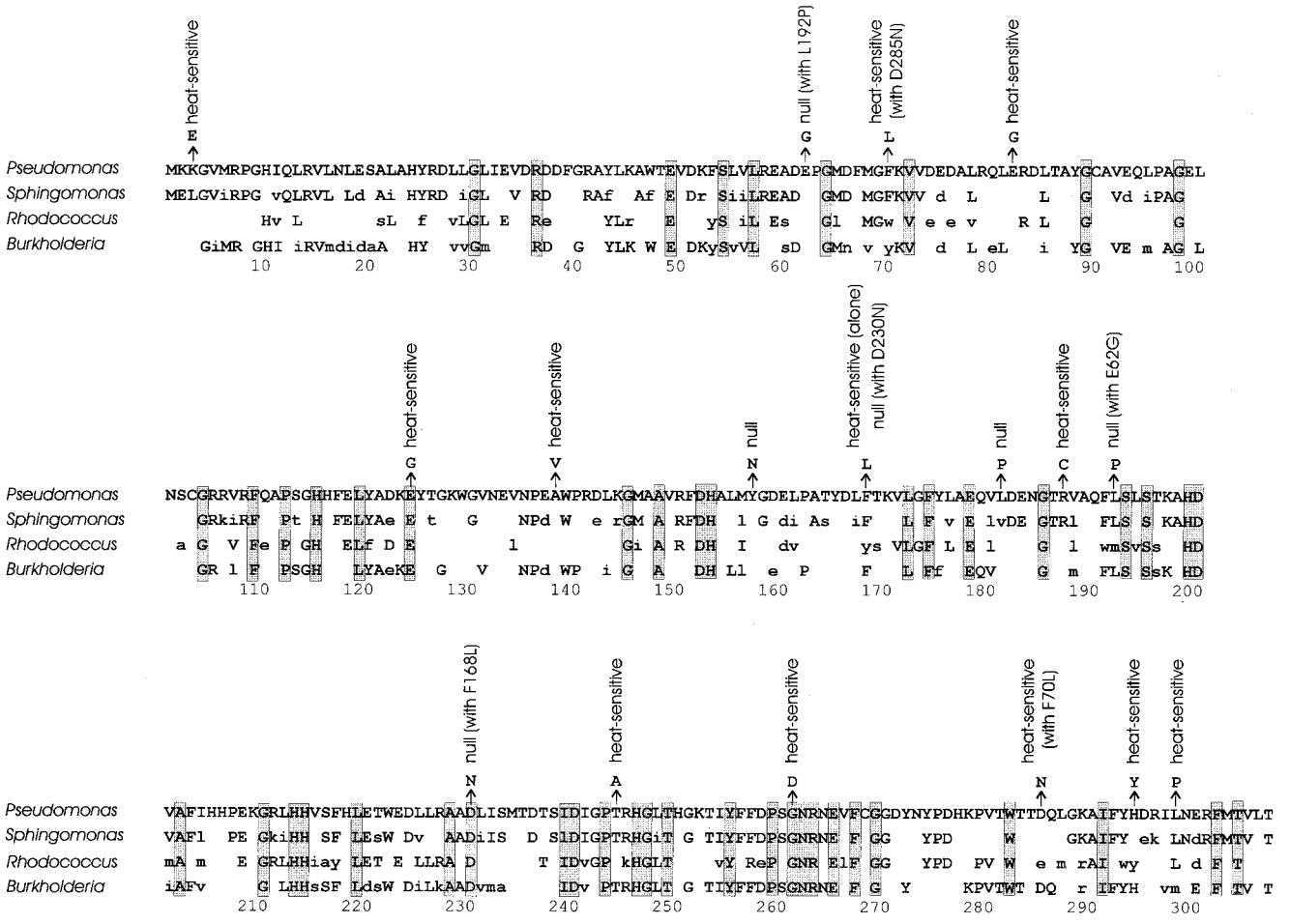

FIG. 4. Comparison of amino acid substitutions causing defects in *Pseudomonas* XylE with amino acids conserved during divergence of XylE in *Sphingomonas* (24), *Rhodococcus* (2), and *Burkholderia* (16). This presentation does not take into account gaps required for optimal alignment. Amino acid residues identical to those in *Pseudomonas* XylE are in uppercase, similar amino acids are in lowercase, and different amino acids are not shown. Shaded boxes mark amino acid residues conserved in all four proteins.

lective basis for the conservation of these residues becomes an open question.

In some cases amino acid substitutions with seemingly minor chemical effects resulted in defective enzymes. Thus, A138V, a conservative substitution at a position where evolutionary divergence had been tolerated, rendered *Pseudomonas* XylE heat sensitive. In two cases, F70L and F168L, substitutions at positions where various aromatic acid substitutions had been tolerated by evolution resulted in heat-sensitive enzymes. The H294Y substitution renders *Pseudomonas* XylE defective by the introduction of an amino acid residue that is maintained in the *Rhodococcus* enzyme.

Limitation of expression of heterologous genes imposed by demands for coding in *Acinetobacter*. A potential limitation of the procedure for analysis of heterologous genes was the possibility that *Acinetobacter*, generally possessing genes with G+C content in the range between 40 and 46%, might be unable to express genes with a relatively high G+C content. For this reason, the *P. putida xylE* gene with a G+C content of 59.6% was selected for investigation. At least in this particular instance, the high G+C content of the inserted wild-type gene presented no obvious barrier to its expression. A limit may have been pushed, however, by *xylE1215*, which causes the substitution of one arginine codon (CGA) by another (CGG) at the position encoding residue 7 within the protein. The usage of CGG is not unusual in *Pseudomonas* genes (wild-type *xylE* already contains three such codons) but is highly exceptional in *Acinetobacter*. A survey of 14 *cat*, *qui*, *pob*, and *pca* structural genes with typical *Acinetobacter* G+C contents revealed that of 191 arginine codons, only two are CGG (18 are CGA). It appears likely that the additional demand for CGG coding imposed by *xylE1215* lowers the level of expression of this gene in *Acinetobacter*. Nevertheless, the expression of wild-type *Pseudomonas xylE* demonstrates that it should be possible to subject genes from a range of biological sources to structure-function analysis as assessed by a variation in activity as a consequence of mutations acquired by PCR mutagenesis. Such variations may include alterations in specificity (11) as well as a loss of function.

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