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Spontaneous Mutations in *pcaH* and -*G*, Structural Genes for Protocatechuate 3,4-Dioxygenase in *Acinetobacter calcoaceticus*†

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Bacteria containing spontaneous null mutations in *pcaH* and -*G*, structural genes for protocatechuate 3,4-dioxygenase, were selected by exposure of an *Acinetobacter calcoaceticus* strain to physiological conditions in which expression of the genes prevents growth. The parental bacterial strain exhibits high competence for natural transformation, and this procedure was used to characterize 94 independently isolated spontaneous mutations. Four of the mutations were caused by integration of a newly identified insertion sequence, IS1236. Many (22 of 94) of the mutations were lengthy deletions, the largest of which appeared to eliminate at least 17 kb of DNA containing most of the *pca-qui-pob* supraoperonic gene cluster. DNA sequence determination revealed that the endpoints of four smaller deletions (74 to 440 bp in length) contained DNA sequence repetitions aligned imprecisely with the sites of mutation. Analysis of direct and inverted DNA sequence repetitions associated with the sites of mutation suggested the existence of DNA slippage structures that make unhybridized nucleotides particularly susceptible to mutation.

Spontaneous mutations, the source of evolutionary change, are not random. Nor are they predictable (16). Since the observation of mutational hot spots (3), it has been apparent that the predisposition for change in DNA is influenced by its own primary sequence (9, 17, 20, 24, 66). DNA sequence repetitions frequently flank the sites where deletion mutations occur (1, 45, 60), and DNA sequence-directed mutations have been implicated in the substitution, addition, or deletion of base pairs (10, 56, 59). Thus, analysis of mutations may help to define the properties of regions where they occurred, but a full understanding of how DNA context influences mutation is far from achieved.

Another approach to understanding the basis for variations in DNA sequence is to analyze mutations that have been selected, directly or indirectly, during evolution. DNA sequences conserved in widely divergent genes are likely to encode essential functions such as amino acid residues required for ligand binding, catalysis, or protein folding. Knowledge concerning these processes has advanced considerably with the revelation of crystal structures in which the function of specific amino acid residues can be discerned (2, 55). Such information facilitates alignment of conserved residues in divergent proteins and indicates some of the constraints that have been placed upon genetic variation in natural populations. Where selection for specific amino acid residues has not been imposed upon DNA primary sequence, the consequences of genetic fluidity may be detected. Even here, change does not appear to have been entirely random. In some cases, genetic variation appears to have been achieved by the acquisition of sequence repetitions that may be detected at the level of either protein (51, 69) or DNA (12, 21, 31, 43, 46, 49, 62).

In sum, the evolution of DNA sequence can be perceived as an interplay between the dictates of protein and gene. The contributions of each are most readily discerned in the framework of the following information. The crystal structure of an enzyme provides a background for understanding how primary sequence influences its function. An evolutionary record suggests how change may have been achieved or constancy may have been maintained in different regions of primary structure. Powerful procedures for analysis of mutations may give indications of the possibilities for variation presented by the DNA. The above criteria for investigation are met by protocatechuate 3,4-dioxygenase and its structural genes, *pcaH* and -*G*, in *Acinetobacter calcoaceticus.*

Protocatechuate 3,4-dioxygenase (Fig. 1) is distributed broadly among microorganisms (18, 53), where it contributes to dissimilation of aromatic growth substrates via the β-ketoadipate pathway (48). The crystal structure of the enzyme from two bacterial sources, *Pseudomonas putida* (47) and *A. calcoaceticus* (64), has been determined. The protein contains nonidentical subunits in equal number: the α subunit (40) is encoded by the *pcaG* gene, and the β subunit (37) is encoded by the *pcaH* gene. The latter subunit binds ferric ion, which contributes to catalytic activity. Ligation of the iron is achieved by two tyrosyl and two histidyl residues, and these amino acids are conserved within the primary structures of widely divergent dioxygenases (32). The α and β subunits of protocatechuate 3,4-dioxygenase share common ancestry and are folded in symmetrical array to form the functional protein in which the α subunit contributes to substrate binding (47). Genes for the respective β and α subunits have been sequenced and are expressed in the order *pcaH* and -*G* in *A. calcoaceticus* (32) and other bacteria (23, 70).

A. calcoaceticus ADP1 exhibits extraordinary competence for natural transformation (38), and this trait allows modified DNA to be either introduced (14) or recovered (29) from the chromosome. The possibility that *A. calcoaceticus* genetics

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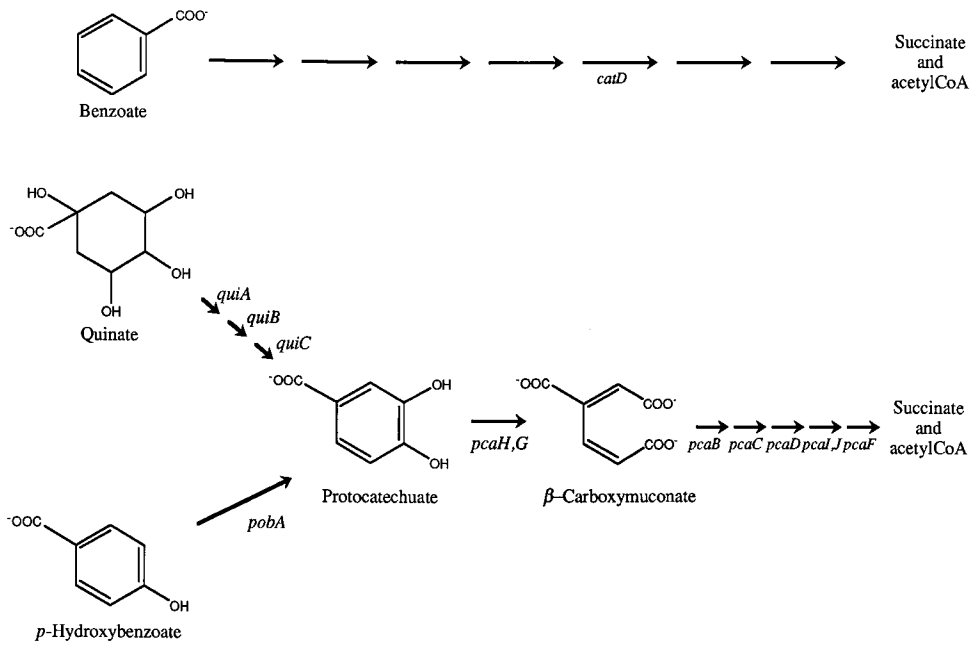

FIG. 1. Role of protocatechuate 3,4-dioxygenase in bacterial metabolism. One set of metabolic reactions, requiring the *qui* genes, gives rise to protocatechuate from quinate. A convergent metabolic reaction, requiring *pobA*, forms protocatechuate from *p*-hydroxybenzoate. Metabolism of protocatechuate in cells that cannot express *pcaB* causes accumulation of β-carboxymuconate to levels that prevent growth with succinate. Thus mutants unable to express *pcaHG* can be selected by exposing them to medium containing 10 mM succinate and 1 mM protocatechuate. The *pcaD* and *catD* genes encode isofunctional enzymes (35, 68). Expression of *catD* is required for growth with benzoate, but this gene can be complemented by *pcaD* which is expressed constitutively in cells containing null *pcaHG* mutations (6). Following selection of *pcaHG* null mutations in strains containing both Δ*pcaBDK1* and Δ*catD101*::Km^r, the Δ*pcaBDK1* deletion can be replaced by providing the corresponding wild-type DNA (as the DNA insert from pZR3) accompanied by selection for growth with benzoate (33).

might be applied effectively to analysis of spontaneous mutations in the *pcaH* and -*G* region emerged from observation of the growth properties of organisms carrying a *pcaBDK* deletion that inactivated PcaB and PcaD, enzymes required for metabolism of the product of protocatechuate 3,4-dioxygenase (41) (Fig. 1). Such organisms do not grow with succinate in the presence of *p*-hydroxybenzoate, the metabolic precursor of protocatechuate. Exposure to *p*-hydroxybenzoate selected strains carrying secondary mutations blocking metabolism of either *p*-hydroxybenzoate or protocatechuate. Replacement of the original *pcaBDK* deletion with wild-type DNA produced strains in which the selected mutations were the sole barrier to growth with the aromatic substrates (34). The location of the selected mutations then could be detected simply by exposing cells to a series of fragments containing nested deletions of wild-type DNA on plates containing *p*-hydroxybenzoate as growth substrate (12, 13).

The purpose of this investigation was to select for and to characterize spontaneous mutations that inactivate *pcaH* and -*G*. Selection procedures were modified to yield solely strains lacking a functional protocatechuate 3,4-dioxygenase. Many of the selected strains were altered in the *pcaH* and -*G* primary sequence, and analysis of the mutations gives insight into mechanisms underlying genetic variation in the protocatechuate 3,4-oxygenase structural genes.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used in this study are listed in Tables 1 and 2. Except where indicated, cultures were grown at 37°C in minimal medium (50) supplemented with 10 mM succinate, 5 mM *p*-hydroxybenzoate, 5 mM quinate, 2.5 mM benzoate, or 10 mM glucose. *Escherichia coli* strains were grown in LB medium (58) supplemented as necessary with ampicillin (75 μg/ml) or tetracycline (12.5 μg/ml).

Transformation of *A. calcoaceticus*. Recipient *A. calcoaceticus* strains were grown overnight in 5 ml of minimal medium containing 10 mM succinate. After addition of 10 μl of 1 M succinate, the cultures were incubated for 30 min and then spread upon plates containing selective growth medium. Donor DNA was dropped on the cell lawn, and transformant colonies appeared after 1 or 2 days incubation at 37°C.

Crude preparations of donor DNA were prepared by lysing donor cells from a 5-ml culture in 0.5 ml of saline-citrate buffer containing 0.05% sodium dodecyl sulfate at 60°C for 1 h (38). Purified DNA or DNA generated by PCR was used in amounts ranging from 0.1 to 1.0 μg. Transformation with plasmids containing chromosomal inserts can lead to recombination of the entire plasmid into the chromosome. Therefore, transforming DNA to be selected after chromosomal insertion was separated from vector DNA by digestion with restriction enzymes. Plasmids to be selected and maintained as independent replicons were not linearized before their introduction by transformation into recipient strains.

Selection of mutant strains unable to express *pcaH* and *G*. Mutations blocking expression of *pcaH* and -*G* were selected in strain ADP500 which carries the Δ*pcaBDK1* and Δ*catD101*::Km^r deletion mutations (33, 41). The Δ*pcaBDK1* mutation prevents metabolism of carboxymuconate and thus prevents growth of cells that accumulate this metabolite from quinate, *p*-hydroxybenzoate, or protocatechuate (Fig. 1). Growth in the presence of *p*-hydroxybenzoate or protocatechuate is made possible only by mutations that block their conversion to

TABLE 1. Bacterial strains

A. calcoaceticus strain	Relevant genotype or phenotype	Reference or source
ADP1	Wild type (strain BD413)	39
ADP6	*pcaG6 pcaG3006*	15
ADP70	*leu3001*	This laboratory
ADP141	*pcaJ3125* Δ*ben-cat3141*	15
ADP239	*pobR10*	13
ADP248	*pobA10*	12
ADP252	*pobA11*	12
ADP500	*pcaG6* Δ*pcaBDK1* Δ*catD101*::Km^r	33
ADP602	Δ*qui-1*::Km^r	19

TABLE 2. Plasmids

Plasmid	DNA insert	Reference or source
Derived from pUC18		67
pZR1	11,062-bp *Eco*RI fragment containing *pcaIJFBDKCHG* operon and *quiBC′*	14
pZR2	2,392-bp *Hin*dIII fragment containing *pcaK′CHG*	32
pZR3	2,754-bp *Hin*dIII fragment containing *pcaB′DK′*	32
pZR211	1,373-bp *Sal*I-*Hin*dIII fragment containing *pcaH′* and *-G*	33
pZR2102	Subclone of pZR211 containing 93-bp deletion	This work (Fig. 3)
pZR2103	Subclone of pZR211 containing 210-bp deletion	This work (Fig. 3)
pZR2105	Subclone of pZR211 containing 314-bp deletion	This work (Fig. 3)
pZR2106	Subclone of pZR211 containing 378-bp deletion	This work (Fig. 3)
pZR2107	Subclone of pZR211 containing 514-bp deletion	This work (Fig. 3)
pZR2108	Subclone of pZR211 containing 667-bp deletion	This work (Fig. 3)
pZR2109	Subclone of pZR211 containing 720-bp deletion	This work (Fig. 3)
pZR2111	Subclone of pZR211 containing 865-bp deletion	This work (Fig. 3)
pZR2113	Subclone of pZR211 containing 983-bp deletion	This work (Fig. 3)
Derived from pUC19 (pZR210)	1,017-bp *Hin*dIII-*Sal*I fragment containing *pcaK′CH′*	33

carboxymuconate (for example, mutations in *pcaH* and *-G*). The *pcaD* and *catD* genes encode isofunctional *A. calcoaceticus* β-ketoadipate enol-lactone hydrolyases which are required for the respective metabolism of protocatechuate and benzoate (35, 68). Because the *pcaD* and *catD* genes encode isofunctional products, DNA containing *pcaD* can be selected in transformants by demanding complementation of the Δ*catD101*::Km^r deletion during growth with benzoate (33).

For selection of strains unable to express *pcaH* and *-G*, single colonies of succinate-grown strain ADP500 were spread upon plates containing 10 mM succinate and 1 mM protocatechuate. This selective medium usually yielded several colonies that grew in the presence of protocatechuate. To assure the isolation of strains carrying independently occurring mutations, only one protocatechuate-resistant strain was selected from each colony of succinate-grown strain ADP500. Three additional sequenced mutations (*pcaH11*, *pcaG11*, and *pcaG19*) emerged from similar selection for growth of mutants containing the Δ*pcaBDK1* deletion with 10 mM succinate but in the presence of 1 mM *p*-hydroxybenzoate (12).

A general assessment of wild-type DNA remaining in the protocatechuate-resistant mutant strains was achieved by using them as donors that might give rise to recombinants from recipient cells carrying different mutations preventing growth with either *p*-hydroxybenzoate or quinate (Fig. 1 and 2). Recombinants that had acquired DNA corresponding to Δ*qui-1* were selected on plates containing 5 mM quinate; the other recombinants were selected on plates containing 5 mM *p*-hydroxybenzoate. The presence of lengthy deletions in the protocatechuate-resistant strains was indicated by their inability to repair the mutations *pcaJ3125*, Δ*qui-1*, *pobR10*, *pobA10*, *pobA11*, *pcaH2*, and *pcaG3006* (Fig. 2). Such strains retained the ability to transform strain ADP70 carrying the *leu-3001* mutation, which prevents growth in the absence of leucine (30).

Additional insight into the length of deletions was given by transformation of the protocatechuate-resistant strains with the 11-kbp *Eco*RI restriction fragment from pZR1; this 11-kbp insert contains all of the structural genes of the *pca* operon (Fig. 2). Recipients that were not transformed by DNA from pZR1 were inferred to have mutations extending outside the *pca* operon.

Donor strains that appeared to lack lengthy deletions were genetically modified for further analysis by replacement of the *pca*Δ*BDK1* deletion with the *Hin*dIII insert from plasmid pZR3 (Fig. 2). Recombinants were selected on plates containing 5 mM benzoate. Growth on this medium was possible because the Δ*catD101*::Km^r mutation in the recombinant strains was complemented by the wild-type *pcaD* gene (Fig. 1). The *pcaD* gene is expressed constitutively in strains carrying null mutations in *pcaH* and *-G* because such dysfunctions cause the endogenous metabolic accumulation of protocatechuate which elicits expression of all of the *pca* genes (6). Since growth with benzoate demands constitutive expression of the acquired *pcaD* gene in recombinants carrying the Δ*catD101*::Km^r mutation, observation of transformant colonies on benzoate suggested that they were blocked in *pcaH* and *-G*. Strains containing *pcaH* and *-G* mutations as the sole barrier to growth with *p*-hydroxybenzoate were further characterized by selection of transformants on this growth substrate after exposure of cells to linearized DNA from pZR2 and its subclones, pZR210 and pZR211 (Fig. 2 and 3). The *Hin*dIII insert in pZR2 contains the wild-type *pcaH* and *-G* genes (Fig. 2), and subclones containing nested deletions allowed mutations to be located rapidly within 200 bp (Fig. 3). This information allowed the design of primers for PCR amplification and DNA sequencing.

Measurement of revertant frequency. After growth of mutant cells from a single colony in 5 ml of minimal medium with 10 mM succinate, the number of revertant cells was determined by spreading aliquots of the culture on selective and nonselective medium. The revertant frequency was the fraction of CFU that gave rise to colonies on the selective medium.

Isolation of DNA. Plasmid DNA was isolated by the alkaline lysis method (5). Chromosomal *A. calcoaceticus* DNA to be used as template for PCR was purified by the following modification of a published method (4). Cells from a 5-ml culture were suspended in 1 ml of 50 mM Tris-HCl (pH 8.0)–20 mM EDTA. Proteinase K and sodium dodecyl sulfate were added to final concentrations of 1 and 2 mg/ml, respectively. The mixture was incubated at 37°C for 10 min in order to allow lysis. Water was added to a final volume of 5 ml. After two extractions with 5 ml of phenol-chloroform-isoamyl alcohol (25/24/1, vol/vol/vol) and one extraction with chloroform-isoamyl alcohol (24/1, vol/vol), the DNA was precipitated with 2 volumes of ethanol and redissolved in 500 μl of TE (10 mM Tris-HCl, pH 8, 1 mM EDTA) buffer containing 10 μg of RNase per ml.

DNA sequence analysis. Cloned double-stranded DNA was sequenced using the Sequenase kit version 2.0 (United States Biochemical, Cleveland, Ohio) and

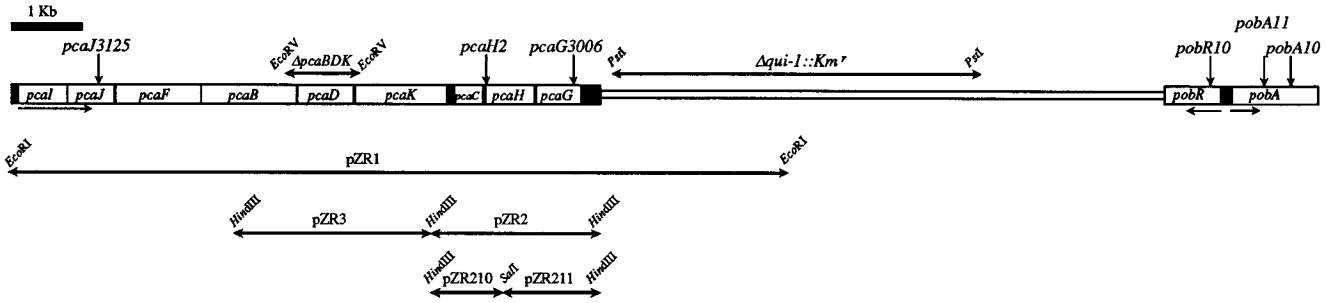

FIG. 2. Map of *A. calcoaceticus* genes associated with metabolism of quinate (*qui*) and *p*-hydroxybenzoate (*pobA*, *pobR*) via protocatechuate (*pcaIJFBDKCHG*). Vertical arrows show the locations of mutations in recipient strains used to locate deletions in donor DNA derived from spontaneous mutant strains. Mapping of the spontaneous mutations was refined by analysis of their ability to form wild-type recombinants after exposure to DNA inserts carried in the indicated recombinant plasmids.

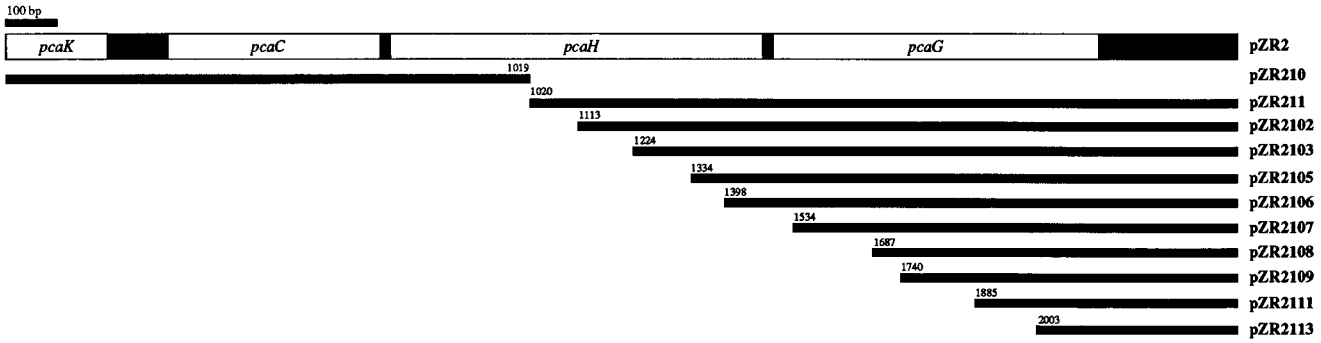

FIG. 3. Locations of the termini of nested deletions used to map *pcaHG* mutations. *A. calcoaceticus* DNA fragments were carried as inserts in pUC19. Nested deletions were derived from pZR211, and all inserts contained DNA fragments ending at the *Hin*dIII site downstream from *pcaG*. The first nucleotide in each nested deletion is indicated by a numeral corresponding to the position of the nucleotide in the DNA sequence of the pZR2 *Hin*dIII fragment as shown in Fig. 4.

either the supplied sequencing primers or custom-designed primers (25). Sequencing of chromosomal DNA was performed after amplification of the relevant locus by PCR. A set of 11 different oligonucleotides (17-mers) corresponding to different sites within the 2.4-kbp *Hin*dIII fragment containing *pcaH* and *-G* served as primers for DNA amplification and sequence analysis. PCR was performed with the DNA Thermal Cycler and AmpliTaq DNA Polymerase (Perkin Elmer Cetus, Wilton, Conn.) under the standard conditions recommended by the manufacturer. Denaturation was assigned 1 min at 94°C, annealing was done for 2 min at 37°C, and polymerization was done for 3 min at 72°C. The reaction was run for 30 cycles with automatic extension of the polymerization step by 5 s per cycle.

DNA amplification for sequence determination was performed in two steps. The first step was a symmetric PCR with 1 μg of chromosomal DNA as template and 100 pmol of each primer in a volume of 100 μl. The second step was an asymmetric reaction using 100 pmol of one primer and 1 pmol of the other primer in 100 μl containing 2 μl of the first reaction as a template in order to produce an excess of the DNA strand to be sequenced. Prior to sequence analysis, the amplified DNA was separated from oil by chloroform extraction and from all small components including oligonucleotides by filtration using Ultrafree-MC Filter Units Low Binding Cellulose 30,000 NMWL, type PLTK (Millipore, Japan). Sequence determination was performed as described for double-stranded DNA.

The nucleotide sequence reported here contains several corrections of the earlier reported DNA sequence for this region (32) and is available under accession no. L05770 from GenBank.

RESULTS

Screening mutant strains for the presence of wild-type DNA. The initial selection for protocatechuate-resistant derivatives of ADP500 yielded 94 independently isolated mutant strains. Strains containing large deletions were identified by assessing the ability of their DNA to transform recipients carrying mutations at different loci in the 18-kbp region containing *pcaH* and *-G* (Fig. 2). Roughly a quarter (22 of 94) of the mutations appeared to be large deletions extending from *pcaH* and *-G* beyond one or more of the other examined alleles. The deletions were of various lengths, and differences in transforming capabilities allowed the large deletions to be classified in six groups (categories I to VI, Table 3). Half (11 of 22) of the large deletions appeared to eliminate all of the tested alleles in the 17 kbp of DNA extending from *pcaJ3125* to *pobA10* (category I, Table 3). There was no predominant pattern indicative of hot spots for deletion within the *pca-qui-pob* region of the chromosome.

The four mutants in category VII (Table 3) appear to contain deletions including the *pcaJ3125* allele and extending upstream into a promoter region required for expression of the *pca* operon. With two exceptions, these mutants and those in categories I to VI did not grow with *p*-hydroxybenzoate after exposure to linearized pZR1 (Table 4). The 11-kbp *Eco*RI fragment in this plasmid contains the 8-kb *pca* operon, 114 bp extending upstream from the *pca* operon, and about 3 kbp of DNA downstream from the *pca* operon. Thus the absence of growth with *p*-hydroxybenzoate after transformation with the *Eco*RI fragment indicates that the recipient contains deletions extending outside the *pca* operon.

Of the 68 remaining mutants (categories VIII and IX, Tables 3 and 4), only 2 did not yield transformants after exposure to linearized pZR1. The two exceptional strains may be blocked in the *pca* promoter region. Consistent with the interpretation that expression of their *pca* operon is blocked, they failed to grow with benzoate after exposure to pZR3 which contains DNA overlapping the Δ*pcaBDK1* deletion.

Only one of the strains containing deletions that appeared to extend outside the *pca* operon (categories I to VI, Tables 3 and 4) acquired the ability to grow with benzoate after exposure to pZR3. In contrast, pZR3 conferred the ability to grow with benzoate upon 53 of 68 mutants in categories VIII and IX (Tables 3 and 4). Mutations in 46 of these 53 recombinants

TABLE 3. Classification of spontaneous mutants as donors of wild-type alleles

Category	No. of mutants in category	Presence (+) or absence (−) of wild-type gene corresponding to:							Minimum length (kb) of deleted DNA
		pcaJ3125	*pcaH2*	*pcaG3006*	Δ*qui-1*	*pobR10*	*pobA11*	*pobA10*	
I	11	−	−	−	−	−	−	−	17
II	1	−	−	−	−	−	+	+	16
III	3	−	−	−	−	+	+	+	15
IV	4	+	−	−	−	−	−	−	16
V	2	+	−	−	−	+	+	+	7
VI	1	+	−	−	+	+	+	+	1.3
VII	4	−	+	+	+	+	+	+	0.0
VIII	5	+	−	+	+	+	+	+	0.0
IX	63	+	+	+	+	+	+	+	0.0

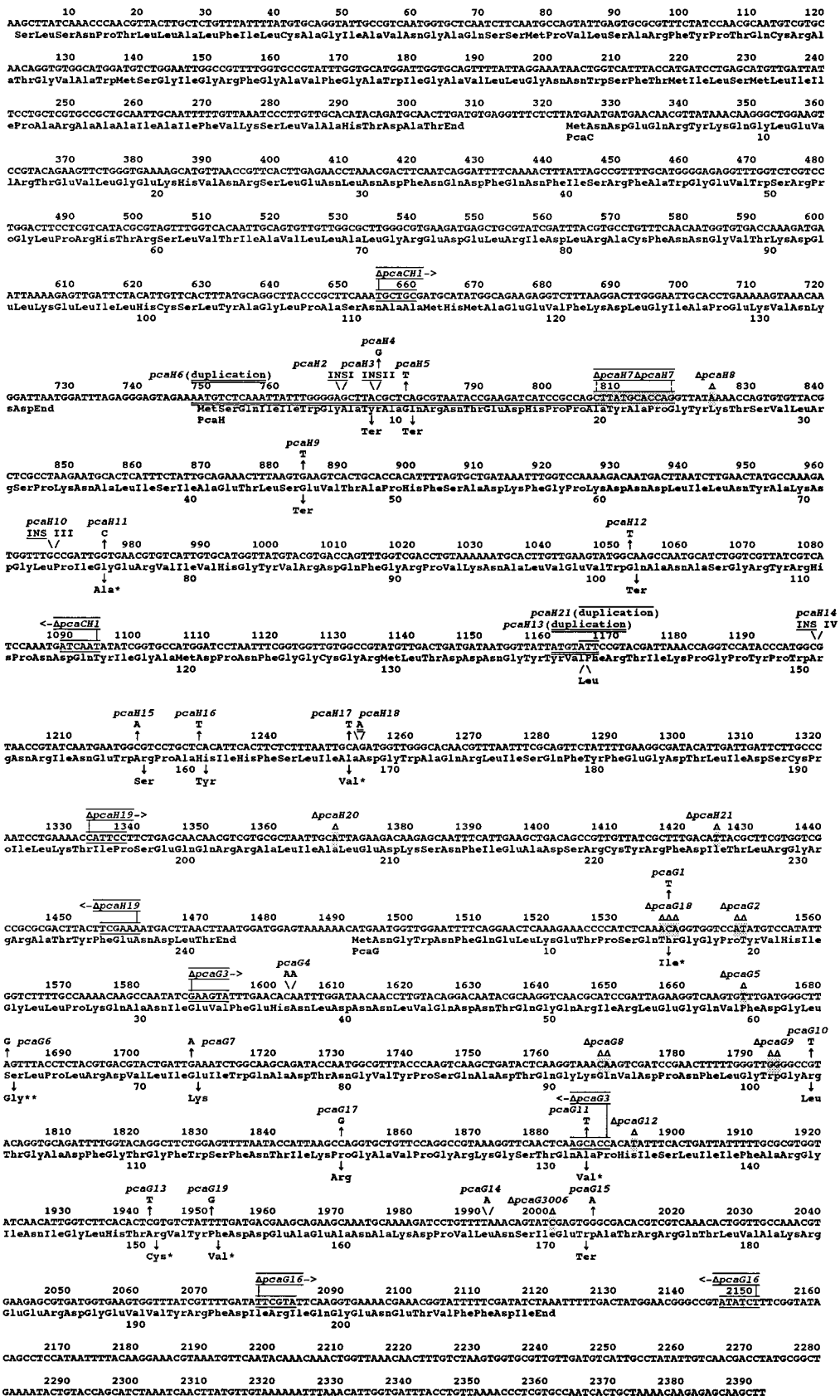

FIG. 4. Locations of spontaneous mutations in coding strand of the 2,392-bp *Hin*dIII restriction fragment containing *pcaHG*. Nucleotide substitutions caused by mutation and corresponding amino acid substitutions are marked by vertical arrows. The ends of lengthy deletions are indicated by overlining and underlining. Nucleotides removed by short deletions are shaded. Double underlining marks sequences tandemly repeated as a consequence of the respective *pcaH6* and *pcaH13* mutations. The 3-bp *pcaH21* duplication shares its locus with *pcaH13* and is both overlined and double underlined. Respective sites where the 1.2-kp insertion sequence IS1236 integrated into the chromosome are marked INS I, INS II, INS III, and INS IV. Nucleotides acquired in short insertion mutations are shown above the DNA sequence.

TABLE 4. Classification of spontaneous mutants as recipients complemented by specific donor DNA fragments

Categories[a]	Growth (mutants grown/total no. of mutants)	
	With *p*-hydroxybenzoate after exposure to DNA insert in pZR1	With benzoate after exposure to DNA insert in pZR3
I–VI	0/22	1/22
VII	2/4	0/4
VIII	5/5	5/5
IX	61/63	48/63

were located within the *pcaCHG* region by demonstration of their ability to grow with *p*-hydroxybenzoate after transformation with the 2,392-bp *Hin*dIII restriction fragment from pZR2 (Fig. 2).

Nested deletions derived from pZR2 (Fig. 3) allowed *pcaH* and *-G* mutations to be mapped with greater precision, and this information was used to select primers for DNA amplification and sequencing of the mutations. As the mutated DNA sequences were identified (Fig. 4), they allowed a measure of the length of overlapping wild-type DNA that is required for recombination to take place. The average length of overlap was 70 bp. The longest overlap, 160 bp, was short enough to allow the mutant nucleotides to be discerned on a single DNA sequencing gel. The shortest overlap required for recombination was 4 bp (Fig. 5).

General properties of spontaneous mutations within *pcaH* and *-G*. DNA sequence modifications associated with each of 42 spontaneous mutations within *pcaH* and *-G* are depicted in Table 5 and Fig. 4. Four of the mutations were caused by insertion of a 1.2-kbp DNA fragment. Each of the DNA inserts yielded the same restriction pattern, and their nucleotide sequence through 100 bp from the site of insertion was the same. Thus it appears that the *Acinetobacter* chromosome contains an insertion sequence, now designated IS1236, whose migration can cause spontaneous mutations. There were seven other insertion mutations all of which were tandem duplications; their respective lengths were 61, 7, 3, 2, (one instance), and 1 (two instances) bp. There were 14 deletion mutations. Four of these exceeded 73 bp in length. The respective lengths of the other deletions were 12, 3, 2 (three instances), and 1 (six instances) bp.

Among the 16 nucleotide substitutions causing dysfunctions in *pcaH* and *-G*, there was a strong preference (15 of 16 cases) for replacement of G · C base pairs. Nine of the nucleotide substitutions were G · C→A · T transitions, three were G · C→T · A transversions, three were G · C→C · G transversions, and one was an A · T→G · C transition. A genetic event that occurred twice is the *pcaG1* mutation, which is a G · C→A · T transition. This mutation lies within the site of the 3-bp Δ*pcaG18* deletion (Fig. 4), and thus this region seems to be particularly susceptible to mutation. Strain ADP6, isolated from the wild-type strain ADP1 in a prior investigation, contains two mutations, *pcaG6* and *pcaG3006*. The wild-type allele corresponding to the latter but not the former mutation is carried in the nested deletion DNA insert of pZR2109. Replacement of *pcaG3006* with DNA from pZR2109 yielded strains retaining *pcaG6*, a result confirmed by DNA sequencing. This mutation, a T · A→G · C transversion, causes substitution of Ser-64 with Gly in the α subunit without apparent

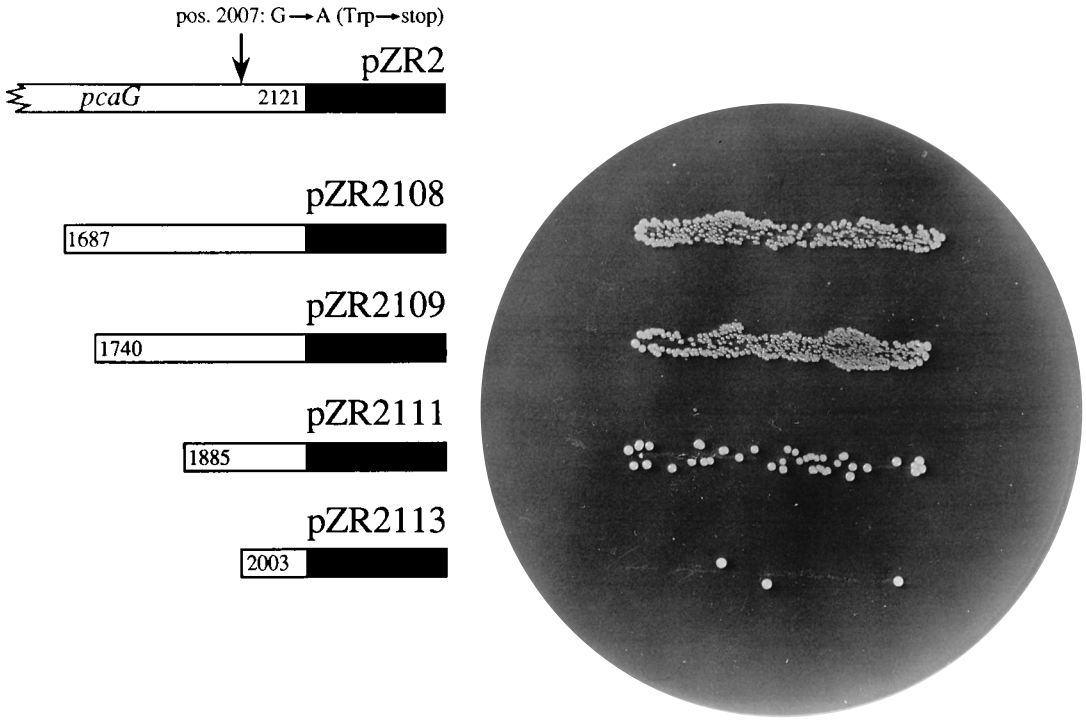

FIG. 5. Assessment of the minimum overlap required for transformation of a mutant allele. The recipient strain containing the *pcaG15* mutation was spread upon a plate containing *p*-hydroxybenzoate as growth substrate, and linearized plasmids containing different nested deletions derived from pZR2 (Fig. 3) were streaked across the plate. All of the nested deletions contained DNA overlapping the locus of the mutation (position 2007) by 385 bp in the downstream direction. Overlap of 122 bp in the upstream direction was sufficient to give rise to frequent transformants (pZR2111), and some transformants were formed when recipients were exposed to DNA containing only a 4-bp overlap in the upstream direction (pZR2113).

TABLE 5. Sequenced mutations in *pcaH* and *-G*

Mutation	Length (bp)	Location in DNA sequence	Substitution	Enzyme functional at:		Strain[a]
				20°C	37°C	
Deletions						
Δ*pcaCH1*	440	655–1094				ADP6315
Δ*pcaG3*	302	1588–1889				ADP6311
Δ*pcaH19*	128	1333–1460				ADP6417
Δ*pcaG16*	74	2078–2151				ADP6348
Δ*pcaH7*	12	807–818				ADP6338
Δ*pcaG18*	3	1537–1539				ADP6504
Δ*pcaG2*	2	1548–1549				ADP6115
Δ*pcaG8*	2	1768–1769				ADP6342
Δ*pcaG9*	2	1793–1794				ADP6127
Δ*pcaH8*	1	824				ADP6312
Δ*pcaH20*	1	1369				ADP6209
Δ*pca21*	1	1425				ADP6242
Δ*pcaG5*	1	1669				ADP6234
Δ*pcaG12*	1	1893				ADP6412
Δ*pcaG3006*	1	2001				ADP6
Tandem insertions						
pcaH6	61	748–808				ADP6212
pcaH13	7	1161–1167				ADP6217
pcaH21	3	1165–1167				ADP6505
pcaG4	2	1602–1603				ADP6237
pcaH18	1	1252				ADP6146
pcaG14	1	1991				ADP6106
Insertions of IS1236						
pcaH2		769–770				ADP6133
pcaH3		774–775				ADP6119
pcaH10		967–968				ADP6409
pcaH14		1199–1200				ADP6108
Nucleotide substitutions causing termination of translation						
pcaH4		774	TAC→TAG			ADP6408
pcaH5		780	CAG→TAG			ADP6314
pcaH9		884	GAA→TAA			ADP6205
pcaH12		1052	CAA→TAA			ADP6407
pcaG15		2007	TGG→TGA			ADP6210
Nucleotide substitutions causing replacement of amino acids						
pcaH11		975	GGT→GCT Gly→Ala	+	−	ADP2509
pcaH15		1220	CGT→AGT Arg→Ser	−	−	ADP6125
pcaH16		1229	CAC→TAC His→Tyr	−	−	ADP6138
pcaH17		1251	GCA→GTA Arg→Ser	+	−	ADP6222
pcaG1		1538	ACA→ATA Thr→Ile	+	−	ADP6116
pcaG6		1681	AGT→GGT Ser→Gly	+	+	ADP6
pcaG7		1708	GAA→AAA Glu→Lys	−	−	ADP6244
pcaG10		1799	CGT→CTT Arg→Leu	−	−	ADP6509
pcaG17		1850	CCA→CGA Pro→Arg	−	−	ADP6501
pcaG11		1886	GCA→GTA Ala→Val	+	−	ADP2507
pcaG13		1942	CGT→TGT Arg→Cys	+	−	ADP6208
pcaG19		1951	TTT→GTT Phe→Val	+	−	ADP6510

[a] Strains with reported designations higher than ADP6000 were isolated as part of this investigation and contain the Δ*catD101*::Km^r mutation. This mutation is not present in reported strains with designations lower than ADP6000.

effect on the overall activity of protocatechuate oxygenase. Additional DNA sequencing revealed that *pcaG6* occurred during preparation of strain ADP500 from the wild-type strain ADP1. Therefore, the mutant strains selected in this investigation contain *pcaG6* maintained from strain ADP500.

Most of the spontaneous *pcaH* and *-G* mutations were stable; only 6 of the 42 examined mutants gave rise to revertants with frequencies exceeding 10^{-8}. Revertants arose with frequencies less than 10^{-7} from one of the nucleotide substitutions (*pcaH4*), from one single-base-pair insertion (*pcaG14*) and from two mutations (*pcaH10* and *pcaH14*) caused by the 1.2-kbp insertion sequence. Substantial instability was observed with two tandem duplication mutations. The 61-bp duplication *pcaH6* yielded revertants with a frequency of 10^{-4}, and a revertant frequency of 10^{-6} was observed with the 7-bp duplication *pcaH13*. The DNA sequences of revertants formed from *pcaH4*, *pcaH10*, and *pcaH13* were identical to those of corresponding loci in the wild-type strain, so true reversion has occurred in these instances.

DISCUSSION

The extent of DNA deleted by spontaneous mutations varies. In light of earlier investigations of spontaneous mutations (1, 59, 60), it was anticipated that many of the selected strains would contain lengthy deletions, and natural transformation was used to estimate the length of deleted DNA. Since use of *A. calcoaceticus* natural transformation for deletion mapping was a novel procedure, it was important to establish the minimum length of donor DNA required to give rise to recombinants. The 338-bp insert in pZR2113 yielded recombinants, and in one instance recombination required an overlap of only 4 bp between donor DNA and recipient allele (Fig. 5). This brief region of sequence identity required for recombination helps to account for the success of mutagenesis of *A. calcoaceticus* by natural transformation with ligated DNA fragments (65). Since natural transformation can detect small DNA segments, it can distinguish between genuine deletions (in which absence of function can be traced to a DNA segment absent in the donor strain) and transpositions (in which function is absent but the corresponding DNA can be rescued from the donor strain). The available evidence indicates that the apparent deletions in every case resulted in loss of DNA from the *A. calcoaceticus* chromosome.

Role of DNA sequence in directing the endpoints of deletions: loops that may help to define regions of hybridization between slipped DNA strands. DNA sequence repetitions frequently flank the endpoints of deletions (1, 9, 45, 59, 60), and it is not surprising that sites of the four lengthiest deletion mutations sequenced in this investigation are marked by flanking DNA sequence repetitions (Fig. 6). Presumably the DNA sequence repetitions allowed hybridization between complementary strands to guide the location of events causing deletion. The repeated sequences do not align precisely with the sites of deletion, and it is reasonable to infer that additional factors may influence such sites.

Realignment of slipped DNA strands would be more likely if they offered multiple opportunities for hybridization after slippage. Indeed, an analysis of *lacI* deletions in *E. coli* led to the suggestion that hybridization of palindromic DNA might form DNA secondary structures that could serve as intermediates in formation of the DNA strand realignments preceding deletion (26, 27, 57). Analysis of other genetic systems has revealed inverted repetitions that might enhance mispairing between extensively slipped DNA strands (9, 45, 66).

Examination of DNA sequences near the sites of *pcaH* and

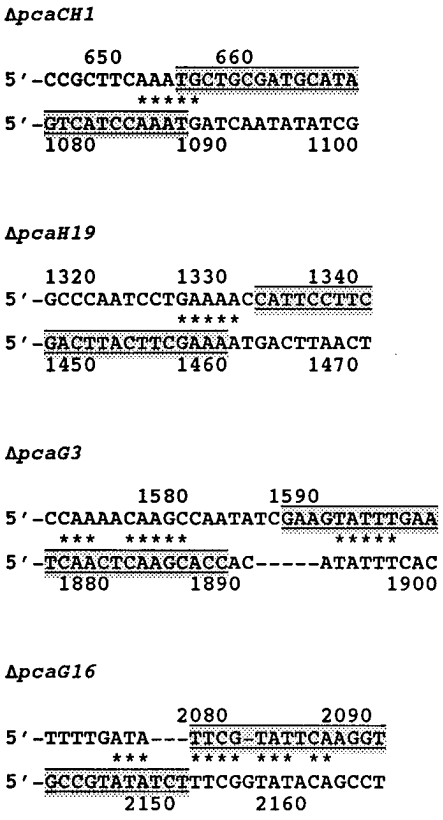

FIG. 6. DNA sequence repetitions flanking the sites of deletions. The coding DNA sequences bordering the sites of lengthy deletions (Fig. 4) are shown. In this and subsequent figures, DNA removed by deletion is shaded, underlined, and overlined. Positions where two or more contiguous nucleotides are identical in aligned DNA sequences from the same strand are marked by asterisks.

-G deletions in *A. calcoaceticus* suggests how multiple DNA strand interactions could help to form secondary structures determining the sites of deletion. An example is shown in Fig. 7, which depicts how loops formed by hybridization between slightly slipped DNA strands could scaffold nucleotides that appear to hybridize after greater slippage. Figure 7A shows the 5 bp presumed to align prior to the 128-bp *pcaH19* deletion (also shown in Fig. 6). Figure 7B indicates how each of the interacting five nucleotide sequences could be framed by hybridization between loops of slightly slipped DNA strands.

DNA slippage structures inferred from the locations of clustered mutations. Within the 1,373-bp *pcaH* and *-G* genes there were 31 sequenced mutations that were either nucleotide substitutions or frameshifts (the duplication or deletion of 3 or fewer base pairs). In three instances (positions 770 to 780, 1537 to 1548, and 1991 to 2007, Fig. 4), mutations were clustered to the extent that three or more occurred within a DNA segment of 17 bp or less. Secondary structures in DNA may account for clustering of these mutations. Those falling between positions 770 and 780 will be discussed below in the context of Fig. 11. The other clusters of mutations are represented in Fig. 8. Figure 8A depicts three localized mutations (*pcaG14*, Δ*pcaG3006*, and *pcaG15*) occurring at nucleotides that are likely to be exposed by intrastrand loops. Likewise, Fig. 8B shows how hybridization between strands after slippage of 12 to 23 nucleotides could form a structure accounting for the occurrence of four independent mutations within a 13-bp DNA sequence. Thus secondary structures formed between

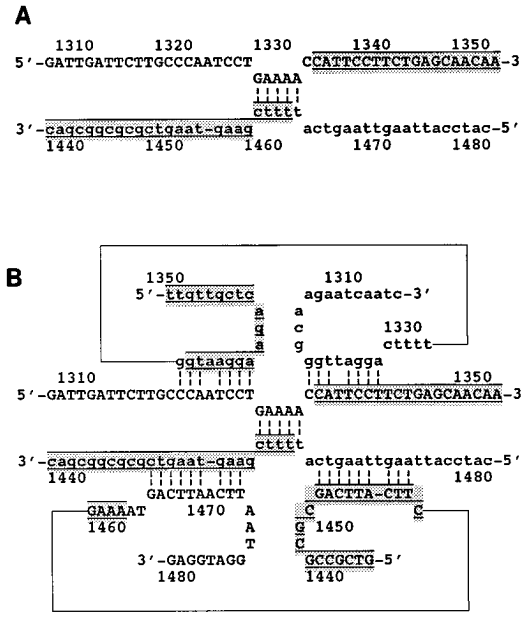

FIG. 7. DNA secondary structures scaffolding potentially hybridizing regions. (A) A 5-bp repetition (5′-GAAAA/3′-ctttt) could allow hybridization of slipped DNA strands near the endpoints of the 128-bp Δ*pcaH19* deletion. (B) Direct repetitions, separated by 13 nucleotides in the upstream sequence and by 17 nucleotides in the downstream sequence, would permit hybridization between slightly slipped DNA strands to frame the 5 nucleotides that are proposed to bring the upstream and downstream sequences together prior to deletion. In this and following figures, solid lines connect contiguous nucleotides forming portions of loops in proposed DNA slippage structures. Dashed vertical lines mark locations where two or more contiguous nucleotides in slipped DNA strands could enter into hybridization. Uppercase letters depict bases in the coding strand, and lowercase letters represent bases in the noncoding strand. Complementary DNA slippage structures, in which the positions of the coding and noncoding strands are reversed, could be formed and thus add to the stability of DNA slippage structures. See the legend to Fig. 6 for more information.

FIG. 8. Potential DNA secondary structures exposing nucleotides that undergo mutation. In this and subsequent figures, the complementary triplets CAC and GTG are highlighted because they frequently flank positions that contribute to the formation of proposed DNA slippage structures. (A) Inverted repetitions between positions 1980 and 2020 may allow loops exposing positions 1992, 2001, and 2008 to changes associated with the respective *pcaG14*, Δ*pcaG3006*, and *pcaG15* mutations. (B) Hybridization between strands after slight slippage (12 to 23 nucleotides) could form a complex loop in which regions undergoing the Δ*pcaG18*, *pcaG1* and Δ*pcaG2* mutations were exposed. It should be noted that *pcaG1* was observed in two independently derived mutant strains (as indicated by the marking 2×). See the legends to Fig. 6 and 7 for more information.

slipped DNA strands may leave unhybridized nucleotides vulnerable to mutation.

Clustered mutations and potential DNA slippage structures at the beginning of *pcaH*. Certain sequences at the beginning of *pcaH* appear to have been freed from major selective constraints imposed at the level of protein. This portion of protein structure is not represented in PcaG nor in any of the other homologous intradiol dioxygenases that have been sequenced (63). As shown in Fig. 9A, few amino acids have been conserved in the NH_2-terminal amino acid sequences of PcaH from *A. calcoaceticus* and *Pseudomonas cepacia* (70). Comparisons of the DNA sequences encoding these portions of protein structure shows that little DNA structure has been conserved from ancestral sequences (Fig. 9B).

A propensity to genetic fluidity is indicated by clustering of mutations in the beginning of *pcaH*. Four of the 42 sequenced mutations lie within the 10-bp region extending from positions 771 to 780 in the wild-type sequence (Fig. 9C). None of these mutations is an amino acid substitution. Since the selection procedure used to isolate spontaneous mutants demanded deficiencies in *pcaH* and *-G* expression, it is likely that additional mutations, causing amino acid substitutions in the NH_2-terminal region of PcaH, existed but were not selected because they did not impair the activity of the protein. Therefore the frequency of observed mutations at the beginning of *pcaH* may give an underestimate of the frequency with which mutations actually occurred. Further evidence for toleration of mutation at the level of protein is presented by the fact that the loss of the tetrapeptide caused by Δ*pcaH724* encoded by the DNA does not completely inactivate the enzyme.

The region of nucleotides 770 to 780, containing the four closely clustered mutations, lies within the 61-bp DNA segment that was duplicated as *pcaH6*, and at the end of this duplication is the 12-bp segment that is deleted by Δ*pcaH7* (Fig. 9C and 10). It is reasonable to propose that the Δ*pcaH7* deletion was preceded by a slippage of 12 bp, and it is notable that alignment after such slippage may account for elimination of a base leading to the Δ*pcaH8* frameshift mutation (Fig. 10). Further involvement of the 12-bp slippage in mutation is suggested by the apparent ability of downstream DNA to form a loop leading to interstrand hybridization with the location where the four clustered mutations occur at positions 770 to 780 (Fig. 11A). Intrastrand hybridization can form additional loops that correlate with these sites of mutation (Fig. 11B and 11C). Thus it appears that freedom from evolutionary constraints at the level of protein allowed DNA to acquire secondary structures suggested by the susceptibility of nucleotides to mutation.

Contributions of the complementary triplets CAC and GTG to DNA slippage structures. Comparison of divergent DNA sequences suggested that the nucleotide triplets CAC and GTG frequently flank DNA segments that may enter into slippage structures (34, 46, 49). Genetic analysis indicated that

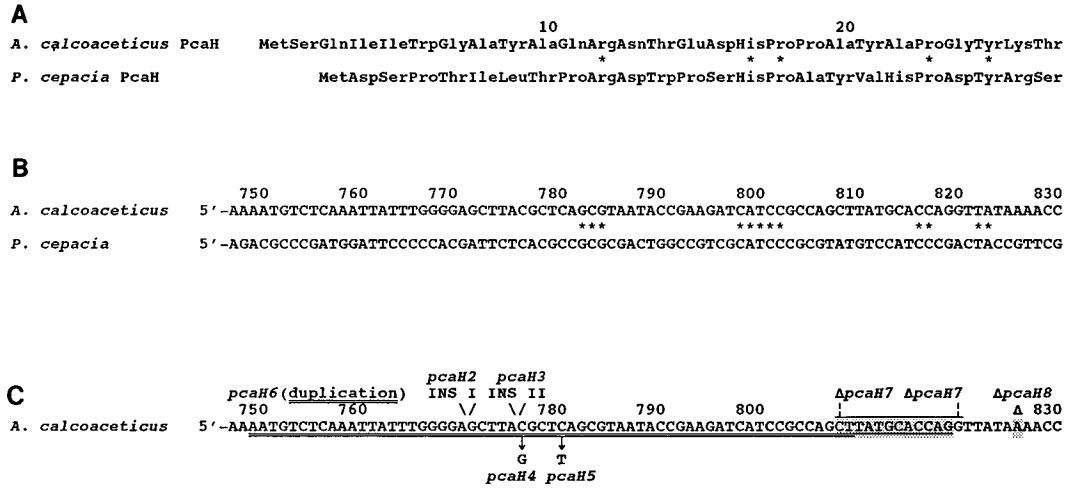

FIG. 9. The location of mutations in the beginning of *pcaH*. (A) Asterisks mark amino acids conserved in the NH_2-terminal amino acid sequence of PcaH from *P. cepacia* and *A. calcoaceticus*. (B) The corresponding aligned DNA sequences contain few similarities, and these can be attributed to codon requirements for the few conserved amino acids. (C) Seven of the 42 sequenced mutations occurred in the first 80 bp of *pcaH*. Four of the mutations are clustered in the 10 bp between positions 770 and 780. In this depiction, the Δ*pcaH7* and Δ*pcaH8* mutations might be regarded as independent, but potential interaction between these mutated regions is shown in Fig. 10. See the legends to Fig. 6, 7, and 8 for more information.

5′-GTGG frequently flanks the sites of frameshifts, deletions, and transpositions in *E. coli lacI* (22). Further evidence for the potential contribution of GTG or CAC to the initiation of DNA strand separation and realignment emerges from identification of these triplets at the sites of loops in the physical structure of DNA (28). The nucleotide triplets contribute to the formation of a bend in DNA at a binding site for the *E. coli* catabolite gene activator protein (61) and are maintained in DNA at a corresponding position in number of different catabolite gene activator protein-binding sites (11). The relative ease with which GTG exchanges protons with solvent suggested that it represents a portion of DNA structure that is readily opened; CAC and GTG often are present at DNA sites associated with transcription, replication, and recombination (44). All of these processes require that a portion of the double helix open into single-strandedness, the same state required for the formation of DNA slippage structures.

The triplets GTG or CAC do not invariably flank DNA sequences that appear to enter DNA slippage structures, perhaps because the contribution of the triplets to strand slippage need not be direct. There is reason to believe that a single region may enter into several different DNA slippage structures, and formation of one DNA slippage structure may trigger establishment of another. Therefore, the potential contribution of GTG or CAC to initiation of a set of DNA slippage structures cannot be discerned until the full set has been identified. Further evidence in this regard is likely to emerge from increased identification of nucleotides that are vulnerable to mutation.

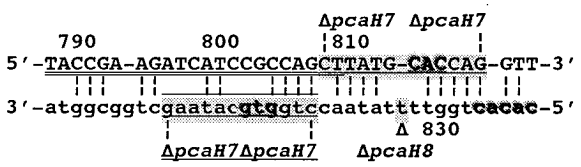

FIG. 10. Slippage of 12 nucleotides can allow an alternatively hybridization pattern accounting for the *pcaH7* deletion. Elimination of a single base pair, experienced in Δ*pcaH8*, would bring the lengths of slipped DNA strands into alignment. See the legends to Fig. 6, 7, and 8 for more information.

Potential selective benefit of DNA sequence repetitions. This investigation was prompted by observation that the acquisition of DNA sequence repetitions appeared to play a major role in the divergence of structural genes for intradiol dioxygenases (32, 46). In light of other studies, it seemed possible that a survey of spontaneous mutations would reveal some that make partial DNA sequence repetitions more perfect (10, 56). Such mutations were not evident among those that caused loss of protocatechuate 3,4-dioxygenase function. The most frequently observed nucleotide substitutions were $G \cdot C \rightarrow A \cdot T$ transitions that can be interpreted as the consequence of the deamination of exposed guanine residues (7, 8).

It must be recognized that examination of null mutations has focused upon the failings of what appears to be a highly evolved set of interactive DNA slippage structures. These structures were acquired during divergence of genes from their evolutionary homologs, and they have been maintained in the absence of significant selection for DNA sequence at the level of protein. It therefore seems possible that the slippage structures are selected at the level of DNA and that a plausible function for the structures is repair (52, 54). Breakdown of this process could result in short DNA sequence duplications observed in this and other (36, 42) investigations. An effective avenue to analysis of the function of DNA slippage structures may be to explore their contribution to reversion of unstable mutations. The unstable chromosomal mutations isolated in this investigation did not revert with discernable frequency when subcloned on plasmids. This evidence alone indicates that the factors governing constancy and change in DNA are influenced by its structure.

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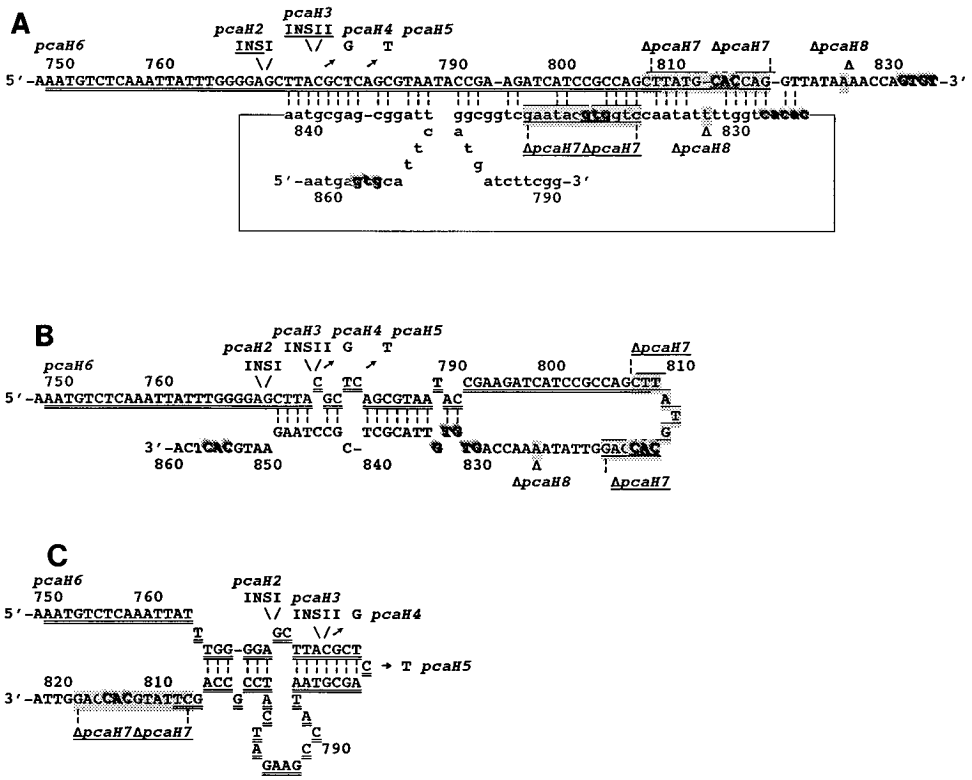

FIG. 11. DNA slippage structures accounting for the sites of mutation in the beginning of *pcaH*. (A) The Δ*pcaH7* deletion (Fig. 10) suggests hybridization that is apparent after slippage of about 12 nucleotides between complementary strands. A loop would allow nucleotides downstream from Δ*pcaH7* and Δ*pcaH8* in the noncoding strand (near position 840, lowercase letters) to hybridize to the upstream region coding strand (uppercase) letters in which the loci of *pcaH2*, *pcaH3*, *pcaH4*, and *pcaH5* are clustered. (B) Inverted repetitions would allow an internal loop in which nucleotides near position 840 could hybridize with DNA containing loci of the four closely clustered mutations. In this hybridization pattern, the clustered mutations occur among the few sites that are not fully hybridized. (C) An additional internal loop has an endpoint near the site containing the termini of the *pcaH6* duplication and the Δ*pcaH7* deletion. See the legends to Fig. 6, 7, and 8 for more information.

REFERENCES

1. **Albertini, A. M., M. Hofer, M. P. Calos, and J. H. Miller.** 1982. On the formation of spontaneous deletions: the importance of short sequence homologies in the generation of large deletions. Cell **29:**319–328.
2. **Allewell, N.** 1989. Evolving to dissimilate hydrocarbons. Trends Biochem. Sci. **14:**473–474.
3. **Benzer, S.** 1961. On the topography of genetic fine structure. Proc. Natl. Acad. Sci. USA **47:**403–415.
4. **Berns, K. I., and C. A. Thomas, Jr.** 1965. Isolation of high molecular weight DNA from *Haemophilus influenzae*. J. Mol. Biol. **11:**476–490.
5. **Birnboim, C., and J. Doly.** 1979. Rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
6. **Cánovas, J. L., and R. Y. Stanier.** 1967. Regulation of the enzymes of the β-ketoadipate pathway in *Moraxella calcoaceticus*. 1. General aspects. Eur. J. Biochem. **1:**289–300.
7. **Coulandre, C., and J. H. Miller.** 1977. Genetic studies of the *lac* repressor. IV. Mutagenic specificity in the *lacI* gene of *Escherichia coli*. K12. J. Mol. Biol. **17:**577–606.
8. **Coulandre, C., J. H. Miller, P. J. Farabaugh, and W. Gilbert.** 1978. Molecular basis of base substitution hotspots in *Escherichia coli*. Nature (London) **274:**775–780.
9. **Dasgupta, U., K. Weston-Hafer, and D. E. Berg.** 1987. Local DNA sequence control of deletion formation in *Escherichia coli*. Genetics **115:**41–59.
10. **DeBoer, J. G., and L. S. Ripley.** 1984. Demonstration of the production of frameshift and base-substitution mutations by quasipalindromic DNA sequences. Proc. Natl. Acad. Sci. USA **81:**5528–5531.
11. **DeCrombrugghe, B., S. Busby, and H. Buc.** 1984. Cyclic AMP receptor protein: role in transcriptional activation. Science **224:**831–838.
12. **DiMarco, A. A., B. A. Averhoff, E. E. Kim, and L. N. Ornston.** 1993. Evolutionary divergence of *pobA*, the structural gene for *p*-hydroxybenzoate hydroxylase in an *Acinetobacter calcoaceticus* strain well-suited for genetic analysis. Gene **125:**25–33.
13. **DiMarco, A. A., B. A. Averhoff, and L. N. Ornston.** 1993. Identification of the transcriptional activator *pobR*, and characterization of its role in the expression of *pobA*, the structural gene for *p*-hydroxybenzoate hydroxylase in *Acinetobacter calcoaceticus*. J. Bacteriol. **175:**4499–4506.
14. **Doten, R. C., L. A. Gregg, and L. N. Ornston.** 1987. Influence of the *catBCE* sequence on the phenotypic reversion of a *pcaE* mutation in *Acinetobacter calcoaceticus*. J. Bacteriol. **169:**3175–3180.
15. **Doten, R. C., K.-L. Ngai, D. J. Mitchell, and L. N. Ornston.** 1987. Cloning and genetic organization of the *pca* gene cluster from *Acinetobacter calcoaceticus*. J. Bacteriol. **169:**3168–3174.
16. **Drake, J. W.** 1991. Spontaneous mutation. Annu. Rev. Genet. **25:**125–146.
17. **Drake, J. W., B. W. Glickman, and L. S. Ripley.** 1983. Updating the theory of mutation. Am. Sci. **71:**621–630.
18. **Durham, D. R., L. A. Stirling, L. N. Ornston, and J. J. Perry.** 1980. Intergeneric evolutionary homology revealed by the study of protocatechuate 3,4-dioxygenase from *Azotobacter vinelandii*. Biochemistry **19:**149–155.
19. **Elsemore, D., and L. N. Ornston.** 1994. The *pca-pob* supraoperonic cluster of *Acinetobacter calcoaceticus* contains *quiA*, the structural gene for quinate-shikimate dehydrogenase. J. Bacteriol. **176:**7659–7666.
20. **Farabaugh, P. J., U. Schmessner, M. Hofer, and J. H. Miller.** 1978. Genetic studies of the *lac* repressor. VII. On the molecular nature of spontaneous hotspots in the *lacI* gene of *Escherichia coli*. J. Mol. Biol. **126:**847–863.
21. **Fieldhouse, D., and B. Golding.** 1991. A source of small repeats in genomic DNA. Genetics **129:**563–572.
22. **Fix, D. F., P. A. Burns, and B. W. Glickman.** 1987. DNA sequence analysis of spontaneous mutation in a PolA1 strain of *Escherichia coli* indicates sequence specific effects. Mol. Gen. Genet. **207:**267–272.
23. **Frazee, R. W., D. M. Livingston, D. C. LaPorte, and J. D. Lipscomb.** 1993. Cloning, sequencing, and expression of the *Pseudomonas putida* protocatechuate 3,4-dioxygenase genes. J. Bacteriol. **175:**6194–6202.
24. **Fuchs, R. P. P., A.-M. Freund, M. Bichara, and N. Koffel-Schwartz.** 1988. DNA structure and mutation hot spots, p. 263–271. *In* R. E. Moses and W. C. Summers (ed.), DNA replication and mutagenesis. American Society for Microbiology, Washington, D.C.
25. **Gerischer, U., and P. Dürre.** 1993. Sequencing using custom designed oligonucleotides, p. 75–81. *In* H. G. Griffin and A. M. Griffin (ed.), Methods in molecular biology. DNA sequencing laboratory protocols. The Humana Press Inc., Totowa, N.J.
26. **Glickman, B. W., P. A. Burns, and D. F. Fix.** 1986. Mechanism of spontaneous mutagenesis: clues from altered mutational specificity in DNA repair

defective strains, p. 159–281. *In* D. M. Shankel, P. E. Hartmen, T. Kada, and A. Hollaender (ed.), Antimutagenesis and anticarcinogenesis, mechanisms. Plenum Press, New York.
27. **Glickman, B. W., and L. S. Ripley.** 1984. Structural intermediates of deletion mutagenesis: a role for palindromic DNA. Proc. Natl. Acad. Sci. USA **81:** 512–516.
28. **Goldberg, I. H., and L. S. Kapen.** 1993. DNA conformation-induced activation of an enediyne for site-specific cleavage. Science **261:**1319–1321.
29. **Gregg-Jolly, L. A., and L. N. Ornston.** 1990. Recovery of DNA from the *Acinetobacter calcoaceticus* chromosome by gap repair. J. Bacteriol. **172:** 6169–6172.
30. **Gregg-Jolly, L. A., and L. N. Ornston.** 1994. Properties of *Acinetobacter calcoaceticus recA* and its contribution to intracellular gene conversion. Mol. Microbiol. **12:**985–992.
31. **Harayama, S., M. Rekik, A. Bairoch, E. L. Neidle, and L. N. Ornston.** 1991. Potential DNA slippage structures acquired during evolutionary divergence of *Acinetobacter calcoaceticus* chromosomal *benACB* and *Pseudomonas putida* TOL pWWO plasmid *xylXYZ*, genes encoding benzoate dioxygenases. J. Bacteriol. **173:**7540–7548.
32. **Hartnett, C., E. L. Neidle, K.-L. Ngai, and L. N. Ornston.** 1990. DNA sequence of genes encoding *Acinetobacter calcoaceticus* protocatechuate 3,4-dioxygenase: evidence indicating shuffling of genes and of DNA sequences within genes during their evolutionary divergence. J. Bacteriol. **172:**956–966.
33. **Hartnett, G. B.** 1993. Ph.D. thesis. Yale University, New Haven, Conn.
34. **Hartnett, G. B., B. Averhoff, and L. N. Ornston.** 1990. Selection of *Acinetobacter calcoaceticus* mutants deficient in the *p*-hydroxybenzoate hydroxylase gene (*pobA*), a member of a supraoperonic cluster. J. Bacteriol. **172:**6160–6161.
35. **Hartnett, G. B., and L. N. Ornston.** 1994. Acquisition of apparent DNA slippage structures during extensive evolutionary divergence of *pcaD* and *catD*, genes encoding identical catalytic activities in *Acinetobacter calcoaceticus*. Gene **142:**23–29.
36. **Huntington's Disease Collaborative Research Group.** 1993. A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's Disease chromosomes. Cell **72:**971–983.
37. **Iwaki, M., H. Kagamiyama, and M. Nozaki.** 1979. The complete amino acid sequence of the β-subunit of protocatechuate 3,4-dioxygenase from *Pseudomonas aeruginosa*. J. Biochem. **86:**1159–1162.
38. **Juni, E., and A. Janick.** 1969. Transformation of *Acinetobacter calcoaceticus* (*Bacterium antitratum*). J. Bacteriol. **98:**281–288.
39. **Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger.** 1988. Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. Gene **70:**191–197.
40. **Kohlmiller, N. A., and J. B. Howard.** 1979. The primary structure of the α-subunit of protocatechuate 3,4-dioxygenase. II. Isolation and sequence of overlapping peptides and complete sequence. J. Biol. Chem. **154:**7302–7308.
41. **Kowalchuk, G. A., G. B. Hartnett, A. Benson, J. E. Houghton, K.-L. Ngai, and L. N. Ornston.** 1994. Contrasting patterns of evolutionary divergence within the *Acinetobacter calcoaceticus pca* operon. Gene **146:**23–30.
42. **Kunkel, T. A.** 1993. Slippery DNA and diseases. Nature (London) **365:**207–208.
43. **Levinson, G., and G. A. Gutman.** 1987. Slipped-strand mispairing: a major mechanism for DNA sequence evolution. Mol. Biol. Evol. **4:**203–221.
44. **Lu, P., S. Cheung, and M. Donlan.** 1985. A recurrent DNA sequence at sites of protein interaction. Adv. Biophys. **20:**153–175.
45. **Mollet, B., and M. Delley.** 1990. Spontaneous deletion formation within the β-galactosidase gene of *Lactobacillus bulgaricus*. J. Bacteriol. **172:**5670–5676.
46. **Neidle, E. L., C. Hartnett, S. Bonitz, and L. N. Ornston.** 1988. DNA sequence of the *A. calcoaceticus* catechol 1,2-dioxygenase I structural gene *catA*: evidence for evolutionary divergence of intradiol dioxygenases by acquisition of DNA sequence repetitions. J. Bacteriol. **170:**4874–4880.
47. **Ohlendorf, D. H., J. D. Libscomb, and P. C. Weber.** 1988. Structure and assembly of protocatechuate 3,4-dioxygenase. Nature (London) **336:**403–405.
48. **Ornston, L. N., and E. L. Neidle.** 1991. Evolution of genes for the β-ketoadipate pathway in *Acinetobacter calcoaceticus*, p. 201–237. *In* K. Towner, E. Bergogne-Berezin, and C. A. Fewson (ed.), The biology of *Acinetobacter*. Plenum Press, New York.
49. **Ornston, L. N., E. L. Neidle, and J. E. Houghton.** 1990. Gene rearrangements, a force for evolutionary change; DNA sequence arrangements, a source of genetic constancy. p. 325–334. *In* K. Drlica and M. Riley (ed.), The bacterial chromosome. American Society for Microbiology, Washington, D.C.
50. **Ornston, L. N., and R. Y. Stanier.** 1966. The conversion of catechol and protocatechuate to β-ketoadipate by *Pseudomonas putida*. I. Biochemistry. J. Biol. Chem. **241:**3776–3786.
51. **Ornston, L. N., and W. K. Yeh.** 1979. Origins of metabolic diversity: evolutionary divergence by sequence repetition. Proc. Natl. Acad. Sci. USA **76:** 3996–4000.
52. **Palmer, B. R., and M. G. Marinus.** 1991. DNA methylation alters the pattern of spontaneous mutation in *Escherichia coli* cells (*mutD*) defective in DNA polymerase III proofreading. Mutat. Res. **264:**15–23.
53. **Parke, D., and L. N. Ornston.** 1984. Nutritional diversity of *Rhizobiaceae* revealed by auxanography. J. Gen. Microbiol. **130:**1743–1750.
54. **Petit, M.-A., J. Dimpfl, M. Radman, and H. Echols.** 1991. Control of large chromosomal duplications in *Escherichia coli* by the mismatch repair system. Genetics **129:**327–332.
55. **Petsko, G. A., G. L. Kenyon, J. A. Gerlt, D. Ringe, and J. W. Kozarich.** 1993. On the origin of enzymatic species. Trends Biochem. Sci. **18:**372–376.
56. **Ripley, L. S.** 1990. Frameshift mutations: determinants of specificity. Annu. Rev. Genet. **24:**189–213.
57. **Ripley, L. S., and B. W. Glickman.** 1983. Unique self-complementarity of palindromic sequences provides DNA structural intermediates for mutations. Cold Spring Harbor Symp. Quant. Biol. **47:**851–861.
58. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
59. **Schaaper, R. M., B. N. Danforth, and B. W. Glickman.** 1986. Mechanism of spontaneous mutagenesis: an analysis of the spectrum of spontaneous mutation in the *Escherichia coli lacI* gene. J. Biol. Chem. **262:**16267–16270.
60. **Schaaper, R. M., and R. L. Dunn.** 1991. Spontaneous mutation in the *Escherichia coli lacI* gene. Genetics **129:**317–326.
61. **Schultz, S. C., G. C. Shields, and T. A. Steitz.** 1991. Crystal structure of a CAP-DNA complex: the DNA is bent by 90°. Science **253:**1001–1007.
62. **Tautz, D., M. Trick, and G. A. Dover.** 1986. Cryptic simplicity in DNA is a major source of genetic variation. Nature (London) **322:**652–656.
63. **van der Meer, J. R., R. I. L. Eggen, A. J. B. Zehnder, and W. M. De Vos.** 1991. Sequence analysis of the *Pseudomonas* sp. strain P51 *tcb* gene cluster, which encodes metabolism of chlorinated catechols: evidence for specialization of catechol 1,2-dioxygenases for chlorinated substrates. J. Bacteriol. **173:**2425–2434.
64. **Vetting, M. W., C. A. Earhart, and D. H. Ohlendorf.** 1994. Crystallization and preliminary X-ray analysis of protocatechuate 3,4-dioxygenase from *Acinetobacter calcoaceticus*. J. Mol. Biol. **236:**372–373.
65. **Vosman, B., R. Kok, and K. J. Hellingwerf.** 1991. Random insertional mutagenesis in *Acinetobacter*, p. 183–189. *In* K. Towner, E. Bergogne-Berezin, and C. A. Fewson (ed.), The biology of *Acinetobacter*. Plenum Press, New York.
66. **Weston-Hafer, K., and D. E. Berg.** 1989. Palindromy and the location of deletion endpoints in *Escherichia coli*. Genetics **121:**651–658.
67. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
68. **Yeh, W. K., P. Fletcher, and L. N. Ornston.** 1980. Evolutionary divergence of coselected β-ketoadipate enol-lactone hydrolases in *Acinetobacter calcoaceticus*. J. Biol. Chem. **255:**6342–6346.
69. **Yeh, W. K., and L. N. Ornston.** 1980. Origins of metabolic diversity: substitution of homologous sequences into genes for enzymes with different catalytic activities. Proc. Natl. Acad. Sci. USA **77:**5365–5369.
70. **Zylstra, G. J., R. H. Olsen, and D. P. Ballou.** 1989. Genetic organization and sequence of the *Pseudomonas cepacia* genes for the alpha and beta subunits of protocatechuate 3,4-dioxygenase. J. Bacteriol. **171:**5915–5921.