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The *pca-pob* Supraoperonic Cluster of *Acinetobacter calcoaceticus* Contains *quiA*, the Structural Gene for Quinate-Shikimate Dehydrogenase†

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An 18-kbp *Acinetobacter calcoaceticus* chromosomal segment contains the *pcaIJFBDKCHG* operon, which is required for catabolism of protocatechuate, and *pobSRA*, genes associated with conversion of *p*-hydroxybenzoate to protocatechuate. The genetic function of the 6.5 kbp of DNA between *pcaG* and *pobS* was unknown. Deletions in this DNA were designed by removal of fragments between restriction sites, and the deletion mutations were introduced into *A. calcoaceticus* by natural transformation. The mutations prevented growth with either quinate or shikimate, growth substrates that depend upon *qui* gene function for their catabolism to protocatechuate. The location of *quiA*, a gene encoding quinate-shikimate dehydrogenase, was indicated by its expression in one of the deletion mutants, and the position of the gene was confirmed by determination of its 2,427-bp nucleotide sequence. The deduced amino acid sequence of QuiA confirmed that it is a member of a family of membrane-associated, pyrrolo-quinoline quinone-dependent dehydrogenases, as had been suggested by earlier biochemical investigations. Catabolism of quinate and shikimate is initiated by NAD^+-dependent dehydrogenases in other microorganisms, so it is evident that different gene pools were called upon to provide the ancestral enzyme for this metabolic step.

Quinate and shikimate are abundant in the biosphere (17), and the ability to use these compounds as growth substrates is distributed broadly among microorganisms (7, 29, 36). In both eukaryotes and prokaryotes, quinate and shikimate are converted to protocatechuate which is metabolized by the β-ketoadipate pathway (Fig. 1). The first enzymatic step in the pathway, the oxidation of either quinate or shikimate, is catalyzed by a single NAD^+-dependent dehydrogenase in members of the eukaryotic genera *Neurospora* (1) and *Aspergillus* (6, 16). Similarly, an NAD^+-dependent dehydrogenase appears to catalyze the first step in quinate catabolism by members of the gram-positive prokaryotic genus *Nocardia* (5).

Initial investigations of quinate catabolism in the gram-negative bacterial species *Acinetobacter calcoaceticus* indicated that quinate dehydrogenase, the product of the *quiA* structural gene (Fig. 1), is a membrane-associated enzyme that acts upon either quinate or shikimate (38). The enzyme is induced by protocatechuate (8), a metabolic product of quinate metabolism (Fig. 1). The electron carrier pyrrolo-quinoline quinone (PQQ) is required for formation of a functional quinate dehydrogenase in *A. calcoaceticus* (23).

Genetic analysis of *A. calcoaceticus* ADP1 is greatly facilitated by its competence for natural transformation (21). This property allowed the use of gap repair (13) to recover a 14.9-kbp chromosomal fragment. A 10.0-kbp subclone (Fig. 2) of the chromosomal fragment extends from *pcaG*, a structural gene for the dioxygenase that acts on protocatechuate (15), to *pobA*, the structural gene for the monooxygenase that converts *p*-hydroxybenzoate to protocatechuate (10). The *pcaIJFBDKCHG* genes and *pobA* were known to be in separate operons because they respond to different metabolite inducers (8, 11). Physical linkage of the genes suggested that they formed part of a supraoperonic cluster of genes with related physiological functions (3). Yet to be determined was the genetic role of the 8.8 kbp of DNA between *pcaG* and *pobA*. More recent investigations have shown that 2.2 kbp of this chromosomal region is occupied by *pobR* (a transcriptional activator of *pobA*) (11) and *pobS* (an apparent repressor of *pobA* expression) (9), which are transcribed divergently from *pobA* (Fig. 2). The function of the approximately 6.5 kbp between the end of *pcaG* and the end of *pobS* was still unknown.

Since protocatechuate is an intermediate in the catabolism of both quinate and shikimate, it seemed that the *qui* genes might lie between *pca* and *pob* in the supraoperonic cluster. An avenue to explore this possibility was afforded by the ease with which genetically engineered deletions can be introduced by natural transformation into the *A. calcoaceticus* chromosome (12). Here we describe the design of deletion mutations that demonstrate the presence of *qui* genes between *pcaG* and *pobA*. Investigation of quinate-shikimate dehydrogenase (QuiA) expression in the deletion mutants allowed localization of *quiA*. A restriction fragment containing *quiA* was cloned, and the DNA sequence of the gene supports the conclusion that it, unlike its counterparts in eukaryotes and gram-positive bacteria, encodes a dehydrogenase that employs PQQ as an electron carrier.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5α was obtained as a frozen suspension of competent cells from Bethesda Research Laboratories (Gaithersburg, Md.). Plasmid constructions were done by using vectors pBSK (Stratagene, Inc., La Jolla, Calif.), pRK415 (22), and pUC18 (43).

Media, growth conditions, and enzyme assays. *E. coli* cultures were grown in Luria broth supplemented as appropriate with ampicillin (75 μg/ml) and tetracycline (12 μg/ml). *A.*

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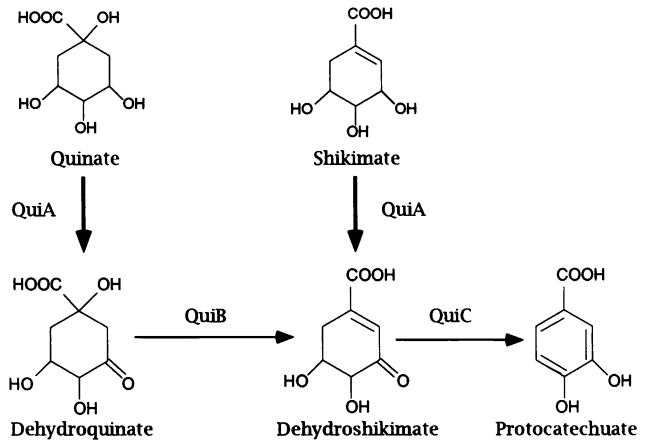

FIG. 1. Metabolic steps converting quinate and shikimate to protocatechuate. A single dehydrogenase, QuiA in *A. calcoaceticus*, oxidizes both quinate and shikimate. QuiB is dehydroquinate dehydratase, and QuiC is dehydroshikimate dehydratase.

calcoaceticus cultures were grown in 200 ml of mineral medium (27) with shaking in 500-ml flasks at 37°C. Carbon sources were supplied at 5 mM, except for succinate, which was provided at 10 mM. Cells were harvested by centrifugation for 10 min at 13,000 × *g*, washed twice with 90 mM sodium phosphate buffer (pH 7.0), suspended in the buffer, and stored at −20°C. Cell extracts were prepared with a Braun-Sonic 2000 sonicator, using three 20-s bursts. Centrifugation at 8,000 × *g* for 10 min in a Microfuge to remove whole cells had no influence on the specific activity of quinate dehydrogenase observed in sonicated cell preparations. The protein concentrations in extracts, as determined by the method of Lowry et al. (24), ranged from 4 to 10 mg/ml. The activity of quinate-shikimate dehydrogenase is associated with cell membranes and was determined by measurement of dichlorophenolindophenol reduction by cell extracts as described by Tresguerres et al. (38). Qualitative observation of protocatechuate accumulation from quinate was made with *E. coli* DH5α(pZR504) colonies spread on plates supplemented with quinate and *p*-toluidine (28).

DNA isolation, manipulation, and sequencing. Routine DNA manipulation was conducted according to published procedures (2, 32). Plasmid DNA was prepared by the boiling-lysis method (19). Subclones for sequencing *quiA* were prepared either with restriction enzymes or by creating a set of

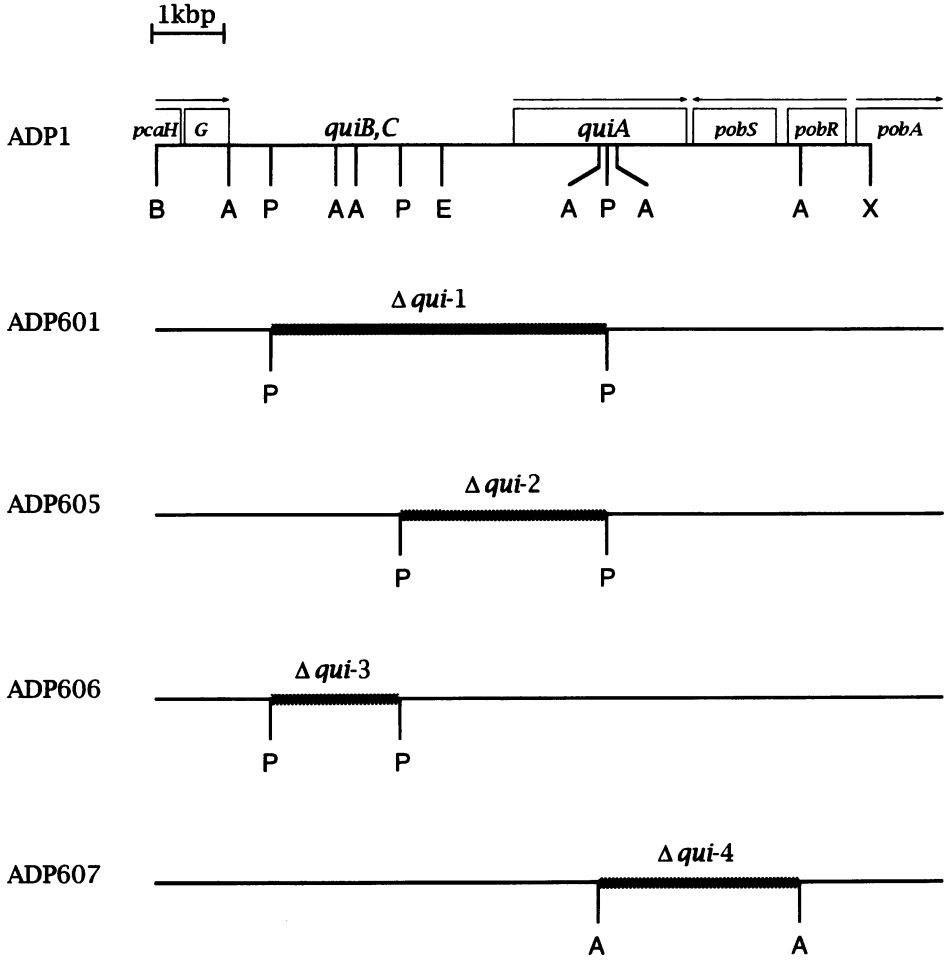

FIG. 2. Schematic representation of a 10-kbp segment of the *A. calcoaceticus* chromosome. Sequenced genes are represented by open boxes, and the directions of transcription are indicated by arrows. Shading marks portions of DNA that were removed by engineered deletions. Restriction enzymes site abbreviations: A, *Acc*I; B, *Bam*HI; E, *Eco*RI; P, *Pst*I; S, *Sac*I; and X, *Xho*I.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
A. calcoaceticus strains		
ADP1	Wild type (strain BD413)	21
ADP601	Δ*qui-1* (chromosomal deletion of 4.8-kbp *Pst*I restriction fragment)	This study (Fig. 2)
ADP605	Δ*qui-2* (chromosomal deletion of 3.0-kbp *Pst*I restriction fragment)	This study (Fig. 2)
ADP606	Δ*qui-3* (chromosomal deletion of 1.8-kbp *Pst*I restriction fragment)	This study (Fig. 2)
ADP607	Δ*qui-4* (chromosomal deletion of 2.7-kbp *Acc*I restriction fragment)	This study (Fig. 2)
ADP617	Δ*qui-3 recA100*::Tn*5*	14; this study
Plasmids		
pBSK	Ap^r	Stratagene
pRK415	Tc^r *lacp/o*	22
pUC18	Ap^r *lacp/o*	43
pZR106	*recA100*::Tn*5* within 11.0-kbp *Eco*RI fragment inserted in pUC19	14
pZR502	pRK415 derivative used to recover *pca-qui-pob* chromosomal DNA by gap repair	3
pZR503	Chromosomal fragment containing wild-type *pca-qui-pob* region as a 14.9-kbp *Hin*dIII-*Sst*I insert in multiple cloning site of pRK415 Tc^r	3
pZR504	pZR503 subclone containing *qui* genes in 10.0-kbp *Bam*HI-*Xho*I DNA fragment in multiple cloning site of pUC18	This study
pZR506	pZR504 subclone with 4.8-kbp *Pst*I restriction fragment deleted (Δ*qui-1*)	This study
pZR509	pZR504 subclone with 3.0-kbp *Pst*I restriction fragment deleted (Δ*qui-2*)	This study
pZR510	pZR504 subclone with 1.8-kbp *Pst*I restriction fragment deleted (Δ*qui-3*)	This study
pZR518	pZR504 subclone containing 6.0-kbp *Eco*I-*Xho*I insert	This study
pZR519	pZR504 subclone with 2.7-kbp *Acc*I restriction fragment deleted (Δ*qui-4*)	This study

nested deletions with exonuclease III. DNA sequencing was performed with Sequenase 2.0 (United States Biochemicals) according to the manufacturer's directions. Oligonucleotides were purchased from the Yale Biotechnology Center. ^{35}S-dATP-labeled samples were run in 8 M urea–6% (wt/vol) acrylamide gels which were dried under vacuum and exposed to Kodak XAR film. Sequence analysis and comparisons were performed with PC/Gene (Intelligenetics, Inc., Madison, Wis.) software and at the National Center for Biotechnology Information with the BLAST network service.

Introduction of designed mutations into the chromosomes of recipient strains. Plasmids with designed deletions in inserts of *A. calcoaceticus* DNA (Table 1) were linearized with *Bam*HI before transformation of recipient cells (12) on succinate plates. After overnight incubation at room temperature, the transformed culture was suspended in basal medium and spread at a dilution that gave rise to single colonies on succinate plates. Picked colonies were examined for their ability to grow on plates with quinate as a growth substrate. Colonies that had lost the ability to grow with quinate occurred with a frequency of between 1 and 10%. The presence of the designed deletion in the recombinant cells was confirmed by restriction mapping of plasmids containing the relevant chromosomal region after their recovery by gap repair (Fig. 3).

Plasmid pZR106 (14) containing a *recA100*::Tn*5* mutation was linearized with *Eco*RI before introduction of the mutation into strain ADP606 (Δ*qui-3*) by natural transformation. Recombinants that had acquired *recA100*::Tn*5* were selected on succinate plates containing kanamycin. The plasmid pZR503, possessing the *pca-qui-pob* region, was introduced by transformation into *E. coli* S17-1 and then transferred into *A. calcoaceticus* ADP617 by conjugation (34); this was followed by selection for growth with succinate in the presence of tetracycline. Transcongugants that grew on this medium had acquired the ability to grow with quinate. Screening for loss of pZR503 from the transcongugants was monitored by examining cells that lost tetracycline resistance after growth from a single colony in a 5-ml succinate culture. Dilutions of the culture were spread on a succinate plate, and colonies that exhibited sensitivity to tetracycline were screened for their ability to grow with quinate.

Nucleotide sequence accession number. The *quiA* nucleotide sequence has been assigned GenBank accession number U11554.

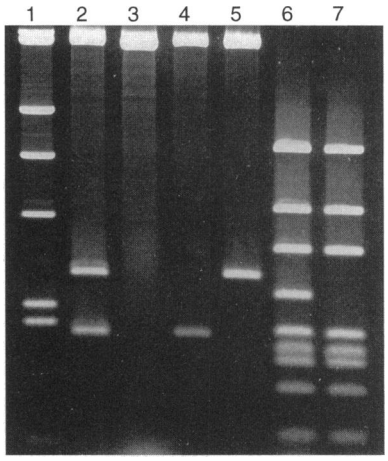

FIG. 3. Restriction analysis of chromosomal fragments recovered by gap repair from *A. calcoaceticus* strains. Lane 1, λ *Hin*dIII standard; lane 2, 1.8- and 3.0-kbp *Pst*I restriction fragments present in DNA recovered from the wild-type ADP1 chromosome; lane 3, both *Pst*I restriction fragments are missing from DNA recovered from strain ADP601 (Δ*qui-1*); lane 4, the 3.0-kbp *Pst*I restriction fragment is missing from DNA recovered from strain ADP605 (Δ*qui-2*); lane 5, the 1.8-kbp *Pst*I restriction fragment is missing from DNA recovered from strain ADP606 (Δ*qui-3*); lane 6, *Acc*I digestion of wild-type DNA yielded a 2.7-kbp fragment; lane 7, the 2.7-kbp *Acc*I fragment is missing from chromosomal DNA recovered from strain ADP607 (Δ*qui-4*).

RESULTS

Preparation and characterization of deletions removing chromosomal DNA between the *pca* and *pob* regions. The *A. calcoaceticus* natural transformation system allows the frequent recovery of recombinants containing DNA that has been modified by genetic engineering (12). Therefore, it was possible to identify the physiological functions associated with specified restriction fragments between *pcaG* and *pobA* by screening the growth properties of recombinants in which the DNA fragments were lacking.

Restriction analysis showed that the 14.9-kbp *Hin*dIII-*Sst*I DNA insert in pZR503 contains an 11-kbp *Hin*dIII-*Acc*I chromosomal segment recovered by gap repair (3). In order to analyze the genetic functions encoded by this segment, a 10-kbp *Bam*I-*Xho*I fragment was subcloned into pUC18, giving rise to pZR504 (Fig. 2). Removal of *Pst*I and *Acc*I restriction fragments from pZR504 formed the deletion mutations Δ*qui-1*, Δ*qui-2*, Δ*qui-3*, and Δ*qui-4* (Fig. 2 and 3), which were introduced into the *A. calcoaceticus* wild-type strain by natural transformation. Transformants that had acquired the deletions lost the ability to grow with quinate or with shikimate. Mutant cells containing Δ*qui-1*, Δ*qui-2*, and Δ*qui-3* grew with *p*-hydroxybenzoate. The Δ*qui-4* deletion extends into *pobR*, and strains containing this mutation did not grow with *p*-hydroxybenzoate.

Expression of *qui* genes in *E. coli* and *A. calcoaceticus*. The 10-kbp *Bam*I-*Xho*I fragment (Fig. 2) was placed downstream of a *lac* promoter in pZR504. Despite the absence of the *lac* repressor in *E. coli* DH5α(pZR504), these cells did not express *quiA* at level permitting the formation of detectable levels of quinate dehydrogenase even after cell extracts had been supplemented with 12 mM PQQ. Low-level expression of the *qui* genes from the plasmid could be inferred because its presence caused protocatechuate to accumulate in *E. coli* DH5α (pZR504) colonies incubated on Luria broth plates supplemented with quinate.

Expression of *qui* genes from pZR503 was apparent because this plasmid allowed growth of *A. calcoaceticus* ADP601(Δ*qui-1 recA100*::Tn*5*) with either quinate or shikimate. The plasmid was lost frequently during growth of cells in the absence of selection for tetracycline resistance. Growth of a 5-ml ADP601 (pZR503) culture from a single-colony inoculum yielded populations in which 50 to 80% of the cells were sensitive to tetracycline. More than 100-tetracycline-sensitive cell lines were screened for their ability to grow with quinate, and in every case loss of this property accompanied loss of the function encoded by the plasmid-borne tetracyline resistance gene.

Mapping of *quiA* and flanking DNA required for quinate catabolism. Of the designed deletions represented in Fig. 2, only Δ*qui-3* allowed chromosomal expression of *quiA*. Full *quiA* expression was achieved during growth of ADP606 (Δ*qui-3*) either with protocatechuate or with *p*-hydroxybenzoate, a compound that is metabolized via protocatechuate. When succinate was added to growth medium containing either protocatechuate or *p*-hydroxybenzoate, QuiA synthesis by either ADP606 or wild-type cells was repressed to about 70% of fully induced levels. Growth with the combination of quinate and succinate allowed QuiA induction in wild-type cells but not in ADP606. Since it has been established that quinate must be metabolized to protocatechuate in order to elicit *quiA* expression (39) and that all of the *qui* structural genes are located between *pca* and *pob*, it could be inferred that the 1.8-kbp *Pst*I fragment deleted from ADP606 encodes other genes associated with quinate catabolism. The Δ*qui-1*, Δ*qui-2*, and Δ*qui-4* deletions prevented expression of *quiA* in response to protocatechuate, and thus it appeared likely that these deletions, unlike the Δ*qui-3* deletion, extended into *quiA*. This inference was explored by determination of the nucleotide sequence of DNA in the region where the endpoints of these deletions overlap (Fig. 2).

Nucleotide sequence of *quiA*. The complete nucleotide sequence of *A. calcoaceticus quiA* is presented in Fig. 4. The 2,427-bp open reading frame is transcribed in the same direction as the other known structural genes in the *pca-qui-pob* supraoperonic cluster. Computer analysis of the deduced amino acid sequence of the *quiA*-encoded quinate-shikimate dehydrogenase indicates that the protein contains five membrane-spanning regions near its amino terminus. The deduced M_r of the protein is 88,196.

DISCUSSION

Genes associated with quinate and shikimate catabolism form part of the *A. calcoaceticus pca-qui-pob* supraoperonic cluster. The results of this investigation demonstrate that the 6.5-kbp DNA segment between *pcaG* and *pobS* in the *A. calcoaceticus* chromosome contains genes required for the metabolic conversion of quinate and shikimate to protocatechuate (Fig. 2). The evidence supporting this conclusion is as follows. Deletion of restriction fragments from the chromosomal region between *pca* and *pob* prevents growth with either quinate or shikimate yet leaves the ability to grow with either protocatechuate or *p*-hydroxybenzoate unimpaired. The 10.0-kbp *Bam*HI-*Xho*I restriction fragment containing DNA bridging *pca* and *pob* conferred upon *E. coli* the capacity, albeit weak, to convert quinate to protocatechuate. The same DNA, carried within the insert of pZR503, complemented the chromosomal Δ*qui-3* deletion by allowing growth with quinate. Loss of pZR503 restored the Δ*qui-3* chromosomal mutants to their phenotypic inability to grow with quinate. The Δ*qui-3* mutation does not prevent expression of *quiA*. The location of this gene, inferred from the locations of Δ*qui-1*, Δ*qui-2*, and Δ*qui-4* (Fig. 2), which prevent *quiA* expression, was proven by determination of its nucleotide sequence.

The mapping of *qui* genes between *pca* and *pob* indicates that the full genetic complement is a *pca-qui-pob* supraoperonic cluster which occupies about 18 kbp in the *A. calcoaceticus* chromosome. Extensive clustering of chromosomal genes with physiologically related functions has been observed before (4, 41, 44) but is unusual among bacteria. Supraoperonic clustering of genes for aromatic catabolism was first reported for fluorescent *Pseudomonas* species (31, 42), but the chromosomal organization of these genes (18) is scattered compared with the tight clustering observed with *pca-qui-pob* and *ben-cat* (33) from *A. calcoaceticus*.

Little information on selective forces that might favor supraoperonic clustering is available. One possibility is that the expression of clustered genes is not completely independent and that transcriptional readthrough allows their unified expression under some physiological circumstances. In some cases, experimental evidence (41, 44) supports this interpretation, but it may not be applicable to the *pca-qui-pob* cluster because the structural genes *quiA* and *pobA* are separated by the divergently transcribed regulatory genes *pobR* and *pobS* (Fig. 2). Furthermore, the expression of *pobA* appears to be tightly controlled and does not respond to protocatechuate, which elicits expression of the upstream structural genes (11).

Another selective force that has been suggested to favor supraoperonic clustering is horizontal transfer of genes with

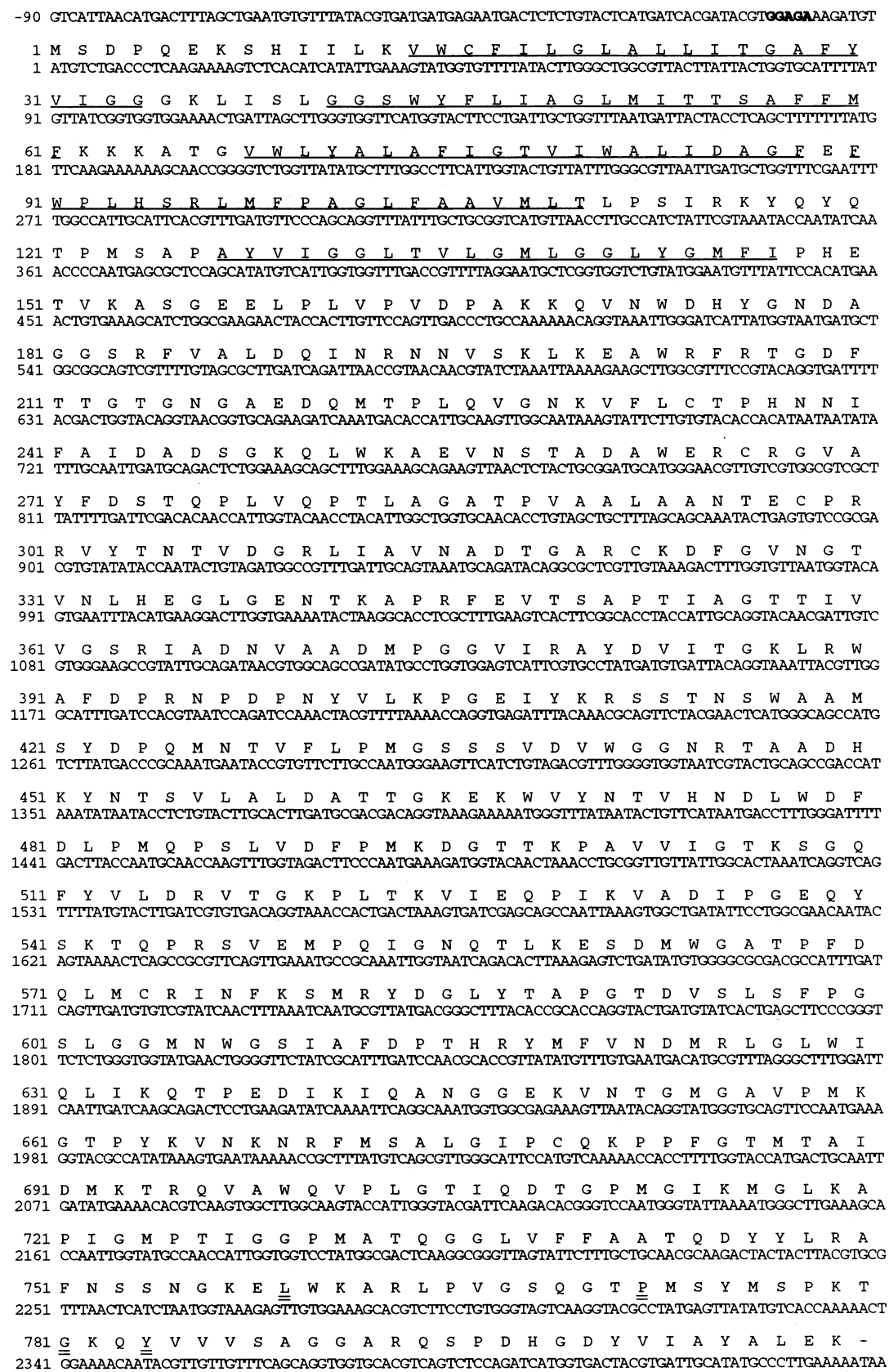

FIG. 4. Nucleotide and deduced amino acid sequences of the *quiA* open reading frame. A possible ribosome-binding site is in boldface, and putative membrane-spanning protein domains are underlined. Residues sharing identity with the PQQ-binding domains of other proteins are double underlined. The termination codon is marked by a dash.

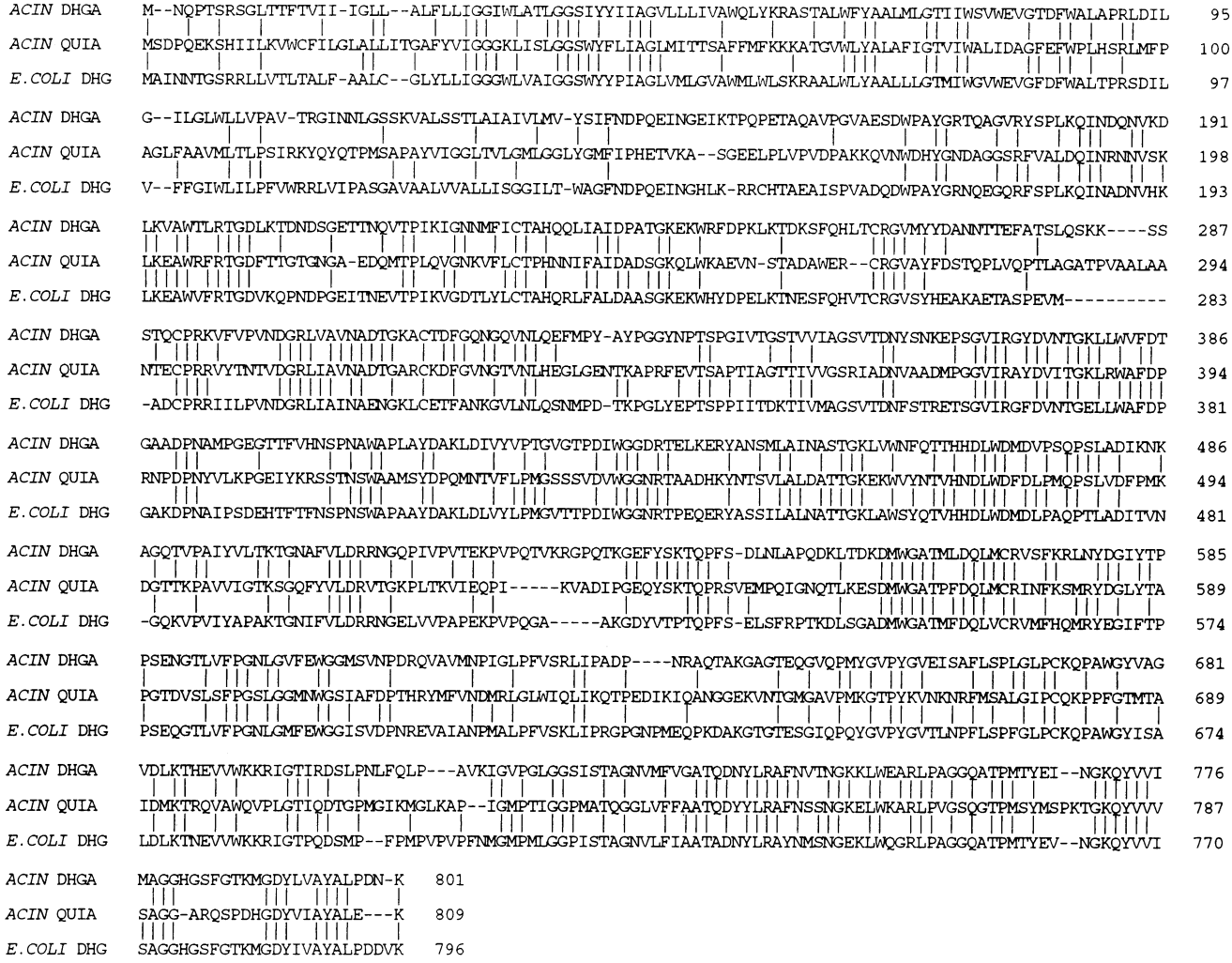

FIG. 5. Vertical lines indicate amino acid residues shared by the primary structure of *A. calcoaceticus* quinate dehydrogenase (*ACIN* QUIA) and the aligned amino acid sequences of glucose dehydrogenase from either *A. calcoaceticus* (*ACIN* DHGA) or *E. coli* (*E. COLI* DHG). The comparison reveals regions of amino acid sequence similarity conserved in the glucose dehydrogenases during evolutionary divergence of the three proteins.

coselected metabolic functions (26, 31, 42). The available evidence indicates that horizontal transfer has not been a dominant force in the recent evolution of *pca-qui-pob* in the *A. calcoaceticus* strain used in this investigation. Our preliminary studies have shown that a number of independently isolated *A. calcoaceticus* strains do not transform mutations in the *pca-qui-pob* cluster, yet these strains can serve as donors for natural transformation of a *trpE* mutation in *A. calcoaceticus* BD413, the parental organism for the strains used in this investigation (21). If failure to transform is taken as a measure of genetic divergence, the evidence suggests that horizontal transfer that might conserve nucleotide sequences in *pca-qui-pob* of *A. calcoaceticus* strains is no more frequent than horizontal transfer of the essential biosynthetic *trpE* gene.

Since there is reason to doubt that either transcriptional readthrough or horizontal gene transfer caused the remarkable clustering within *ben-cat* and *pca-qui-pob*, it would be appropriate to explore how gene rearrangement influences the function and stability of genes in these regions. The competence of *A. calcoaceticus* BD413 (strain ADP1) for natural transformation should facilitate such investigations. It also will be of interest to determine the extent to which supraoperonic clustering of the catabolic genes is conserved in other *A. calcoaceticus* strains, most of which do not exhibit a capacity to undergo natural transformation (20).

Nucleotide sequence and deduced amino acid sequence of *quiA*. The deduced *A. calcoaceticus* QuiA amino acid sequence supports the earlier conclusion, based upon biochemical evidence (23), that the protein is a member of a family of PQQ-dependent dehydrogenases. Comparison with known members of the family reveals that *A. calcoaceticus* most closely resembles glucose dehydrogenases from *A. calcoaceticus* and *E. coli* (Fig. 5). The glucose dehydrogenases share amino acid sequence identity of 61.7%; the respective amino acid sequence identities shared by the *A. calcoaceticus* and *E. coli* glucose dehydrogenases with *A. calcoaceticus* QuiA are 32.1 and 37.3% (Fig. 5). This evidence is consistent with the view that the divergence of quinate and glucose dehydrogenases preceded the divergence of glucose dehydrogenases of *A. calcoaceticus* and *E. coli*. More distant divergence from *A. calcoaceticus* QuiA has been experienced by PQQ-dependent dehydrogenases associated with oxidation of ethanol, metha-

nol, and aldehydes, yet all of the PQQ-dependent enzymes retain discernible homology, and the essential nature of some amino residues is demonstrated by their conservation in all members of the enzyme family.

The failure of *E. coli* to express *quiA* at directly detectable levels cannot be attributed to an inability of the organism to form a functional protein using the electron acceptor PQQ. The amino acid sequence similarity of *A. calcoaceticus* QuiA and the PQQ-dependent glucose dehydrogenase of *E. coli* suggests that expression of the *A. calcoaceticus* enzyme does not require radical departure of *E. coli* from physiological traits acquired in its evolutionary experience. It is possible that low-level expression of *quiA* in *E. coli* results from failure to cleave a signal peptide, as has been observed before with PQQ-dependent aldehyde dehyrogenase (37).

Separate ancestries of genes with the same metabolic function. Since its elucidation, the β-ketoadipate pathway has served as a model of patchwork evolution in which different genes were brought into physical proximity as they made interdependent contributions to complex metabolic units of function (26, 27, 35). More recent evidence makes it possible to discern the evolutionary origins of genes representing the different patches (30, 40). In some instances, different gene pools were called upon to provide enzymes achieving analogous metabolic transformations in different organisms. For example, conversion of β-carboxy-*cis,cis*-muconate to β-ketoadipate proceeds by different metabolic mechanisms in prokaryotes and eukaryotes (27), and the participating enzymes in the two biological groups can be traced to different ancestries (25).

A different biological division is suggested by gene pools called upon for the catabolic oxidation of quinate and shikimate. The presently available evidence indicates that NAD^+-dependent dehydrogenases serve this function in eukaryotes (1, 6, 16) and gram-positive bacteria (5), whereas gram-negative bacteria, represented by *A. calcoaceticus*, employ the PQQ-dependent QuiA to initiate catabolism of quinate and shikimate.

Natural transformation as an aid in elucidating physiological functions associated with DNA fragments. This investigation was facilitated by the high frequency with which DNA containing designed deletions recombined into the chromosome as a consequence of natural transformation. The power of this technique should be kept in mind as improvements in physical mapping bring forth DNA segments with unknown physiological functions. As demonstrated here and earlier (12, 14), DNA fragments from *A. calcoaceticus* can be modified and then restored to the chromosome with high frequency by direct addition to recipient cells. Analysis of the recombinants provides evidence for the functions that have been altered as a consequence of the genetic modifications. The extension of these procedures may minimize mysteries associated with open reading frames revealed by chromosomal sequencing.

ACKNOWLEDGMENTS

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