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Cloning and Genetic Characterization of *dca* Genes Required for β-Oxidation of Straight-Chain Dicarboxylic Acids in *Acinetobacter* sp. Strain ADP1†

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A previous study of deletions in the protocatechuate (*pca*) region of the *Acinetobacter* sp. strain ADP1 chromosome revealed that genes required for utilization of the six-carbon dicarboxylic acid, adipic acid, are linked to the *pca* structural genes. To investigate the genes involved in adipate catabolism, a 33.8-kb *Sac*I fragment, which corrects a deletion spanning this region, was cloned. In addition to containing known *pca*, *qui*, and *pob* genes (for protocatechuate, quinate, and 4-hydroxybenzoate dissimilation), clone pZR8000 contained 10 kb of DNA which was the subject of this investigation. A mutant strain of *Escherichia coli* DH5α, strain EDP1, was isolated that was able to utilize protocatechuate and 4-hydroxybenzoate as growth substrates when EDP1 cells contained pZR8000. Sequence analysis of the new region of DNA on pZR8000 revealed open reading frames predicted to be involved in β-oxidation. Knockouts of three genes implicated in β-oxidation steps were introduced into the chromosome of *Acinetobacter* sp. strain ADP1. Each of the mutants was unable to grow with adipate. Because the mutants were affected in their ability to utilize additional saturated, straight-chain dicarboxylic acids, the newly discovered 10 kb of DNA was termed the *dca* (dicarboxylic acid) region. Mutant strains included one with a deletion in *dcaA* (encoding an acyl coenzyme A [acyl-CoA] dehydrogenase homolog), one with a deletion in *dcaE* (encoding an enoyl-CoA hydratase homolog), and one with a deletion in *dcaH* (encoding a hydroxyacyl-CoA dehydrogenase homolog). Data on the *dca* region should help us probe the functional significance and interrelationships of clustered genetic elements in this section of the *Acinetobacter* chromosome.

Microbial β-oxidation of fatty acids has enjoyed prolonged research interest, yet the genetics and biochemistry of dicarboxylic acid catabolism have received minimal attention. The latter acids are of particular interest because they have the potential to play a significant role in the natural environment by serving as cross-linkers between other compounds. In addition, saturated, straight-chain dicarboxylic acids or their thioesters arise as intermediates in catabolic pathways for diverse compounds. Adipic acid is an intermediate in the metabolism of cyclohexanol (14), and other dicarboxylic acids form during oxidation of the corresponding cyclic alcohols. Additional catabolic pathways include ω-oxidation of fatty acids (31), alkane oxidation (29), aerobic degradation of cyclohexanecarboxylic acid (6), and anaerobic metabolism of aromatic compounds such as benzoate, which generates pimelyl coenzyme A (pimelyl-CoA) as an intermediate (22).

Straight-chain dicarboxylic acids of 6 to 10 carbon atoms in length serve as carbon sources for aerobic growth of diverse microbial strains (4, 37, 42). In *Acinetobacter* spp. (4), as in other bacteria characterized for the trait, the ability to utilize saturated dicarboxylic acids of this size range aerobically is often a unit characteristic (23). Experimental evidence with *Pseudomonas fluorescens* supported the hypothesis that this unit trait is a consequence of cyclic β-oxidation steps analogous to those of fatty acid degradation (23).

In the naturally transformable *Acinetobacter* sp. strain ADP1, also designated strain BD413 (28), there is a remarkable, extended cluster of genes for related function in one region of the chromosome, an "island of catabolic diversity" (35). Downstream from 10 genes required for protocatechuate catabolism are genes for conversion of diverse hydroaromatic and aromatic compounds to protocatechuate (Fig. 1). A positive selection strategy for mutations that protect against accumulation of a toxic intermediate in protocatechuate catabolism has been used to study *Acinetobacter* sp. strain ADP1 proteins and regulatory sequences which are required for generating the toxic compound. In one study, a quarter of the spontaneous mutations were deletions, and some of them extended into neighboring genes (18). The discovery that some of the deletions upstream of the *pca* structural genes eliminated the ability of strains to grow on the six-carbon dicarboxylic acid, adipic acid, provided the first evidence for linkage of adipate utilization genes and *pca* genes (10, 11).

This communication describes the cloning and initial characterization of a cluster of open reading frames defined as *dca* genes because of their role in the dissimilation of an array of straight-chain, saturated dicarboxylic acids. Particular emphasis was placed on three genes that were predicted to be required for the central steps of β-oxidation.

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MATERIALS AND METHODS

Source of dicarboxylic acids and their nomenclature. Sigma Chemical Co. was the source of all dicarboxylic acids except tridecanedioic and dodecanedicarboxy-

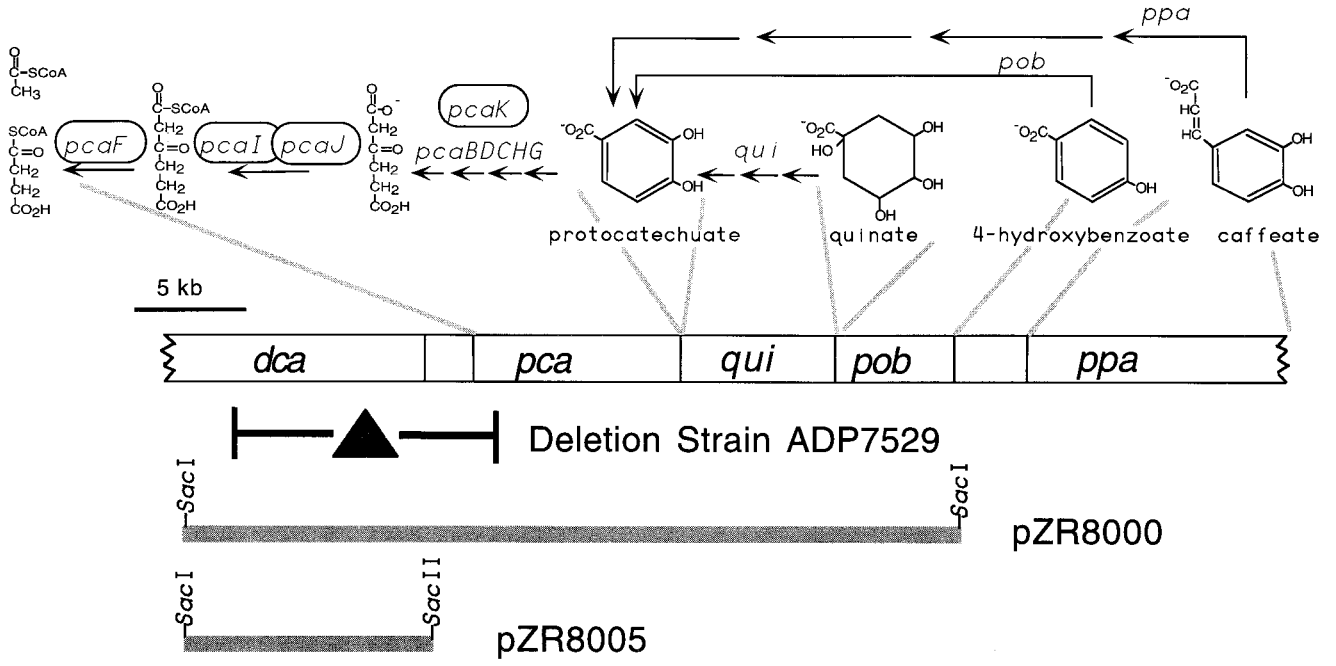

FIG. 1. Relevant strain and plasmids used to clone and sequence DNA adjacent to the *pca* operon from strain ADP1. Gene designations are *dca*, dicarboxylic acid; *pca*, protocatechuate; *qui*, quinate and shikimate; *pob*, 4-hydroxybenzoate; and *ppa*, phenylpropenoid and phenylpropanoid. Above the layout of genetic sections, delineated by the gray lines, are the outlines of pathways for dissimilation of aromatic and hydroaromatic compounds. Genes required for the illustrated biochemical transformations are shown above the related arrows. Those encircled have homologs among the *dca* genes. The deletion within strain ADP7529 was identified as part of this study.

lic acids, which were obtained from Aldrich Chemical Co. Common names are used for the shorter dicarboxylic acids: adipic (6 carbons), pimelic (7 carbons), suberic (8 carbons), and sebacic (10 carbons) acids. Nomenclature for the less familiar acids is dodecanedioic (12 carbons), tridecanedioic (13 carbons), tetradecanedioic (14 carbons), and hexadecanedioic (16 carbons) acids. The dicarboxylic acids were acquired a short time before use, and all of the longer dicarboxylic acids were purported to be 99% pure except tridecanedioic acid, at 94%.

Strains, media, and growth of cells. Strains and plasmids are listed in Table 1. Cells were cultured in Luria-Bertani medium (41) or minimal medium (36). Solidified minimal medium contained adipate at 5 mM or succinate at 10 mM. In some instances, modified gradient plates were used to screen *Acinetobacter* cells for substrate utilization patterns (36): cells were spread on agar-solidified minimal medium, and the carbon source was applied to one spot at the edge of the plate, providing a concentration gradient.

To screen *Acinetobacter* cells for antibiotic resistance markers, a kanamycin concentration of 15 $\mu g\ ml^{-1}$ was used in Luria-Bertani medium, and ampicillin at 110 $\mu g\ ml^{-1}$ was used in minimal medium. When *Escherichia coli* cells were under selection, Luria-Bertani medium was supplemented with chloramphenicol, kanamycin, or ampicillin at 20, 25, or 90 $\mu g\ ml^{-1}$, respectively.

For all tests involving relative yields of *Acinetobacter* strains in liquid medium, stock solutions of substrates were prepared in dimethyl sulfoxide (DMSO) at a concentration of 0.5 M or 0.25 M, as required by solubility. Adipic acid was added to liquid minimal medium at a final concentration of 2 mM. For each of the longer-chain dicarboxylic acids, the final concentration of carbon atoms was equivalent to that provided by 2 mM adipic acid. Controls contained only DMSO, provided at the maximum amount added with any carbon source.

Comparative growth tests of *Acinetobacter* strains were carried out by growing cells overnight at 37°C in minimal medium containing succinate as the sole carbon source. A 50-μl aliquot of an overnight culture of cells was added to 5 ml of fresh minimal medium containing a carbon source, with the inoculum size designed to minimize the contribution of possible revertants to growth. Cultures were incubated at 37°C and 250 rpm. Growth of the wild-type strain was monitored, and the density of each mutant strain on a particular substrate was measured when wild-type cells, grown in parallel on the same substrate, attained their maximum level of growth.

Doubling times on particular substrates were determined by measuring turbidity after inoculating an overnight culture into 10 ml of minimal medium in a 50-ml Erlenmeyer flask and shaking at 37°C and 250 rpm.

Analysis of the ability of *E. coli* cells bearing pZR8000 to grow at the expense of various compounds was tested on solidified medium and in liquid minimal medium, both supplemented with 0.1% yeast extract and chloramphenicol. In this case, substrates were prepared in water and neutralized with sodium hydroxide. A positive control for growth was glucose, and negative controls were sodium chloride or no addition. Since some tested compounds might be toxic at high concentrations, they were provided on gradient plates. Cells were streaked for single colonies across half of each plate, and 70 to 100 μmol of substrate was distributed evenly in a line in the middle of the other half of the plate, perpendicular to the bacterial streaks. Liquid cultures contained substrate at a concentration of 5 mM, and turbidity was determined after 2 days in a 37°C shaker at 250 rpm. Turbidity was measured at 600 nm, and the correlation between increased turbidity and cell growth was confirmed by performing viable counts, particularly important with protocatechuate-supplemented medium, which had a colored tint.

Analysis of revertant frequencies. The apparent revertant frequency of each mutant strain was measured by spreading aliquots of an overnight culture onto minimal medium plates containing adipate as the carbon source. Viable counts of the original cultures were determined on nonselective medium. The fraction of the total viable count that appeared as CFU on the selective medium was taken as the presumptive revertant frequency.

Construction of plasmids and mutant strains. Standard techniques were used in molecular biology manipulations (2, 39). Natural transformation of *Acinetobacter* strains followed published methods (27). The ability of mutant strains of ADP1 to take up DNA carried on plasmids in *E. coli* by replica plating has been demonstrated (3). Competent *E. coli* DH5α cells (26) were transformed with a *Sac*I library of *Acinetobacter* sp. strain ADP1 in vector pBBR1MCS. *E. coli* transformants were replica plated onto a lawn of strain ADP7529 which had been made competent for natural transformation. An *E. coli* colony that transformed the deletion strain to an adipate-positive phenotype was isolated off a master plate, and plasmid pZR8000 was purified from the *E. coli* cells. DH5α(pZR8000) (Fig. 1) also transformed ADP992, a *pobA*-defective strain, to a 4-hydroxybenzoate-positive phenotype.

Strains ADP8018 (Δ*dcaH1*), ADP8023 (*dcaA1*::Km^r), ADP8061 (Δ*dcaA2*), and ADP8062 (Δ*dcaE2*) were created by transformation of the competent parental strain with plasmid pZR8044, pZR8053, pZR8075, or pZR8077, respectively (Table 1; Fig. 2 and 3), followed by phenotype assessment on minimal medium plates containing adipate; the strains were also screened for the absence

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Genotype	Reference or source
E. coli strains			
DH5α	F$^-$ ϕ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17* (r_K^- m_K^-) *supE44* λ^- *thi-1 gyrA96 relA1*		Gibco-BRL
EDP1	Mutant of strain DH5α which degrades 4-hydroxybenzoate when it carries pZR8000		This study
Acinetobacter sp. strains			
ADP1	Wild-type strain (BD413)		28
ADP992	ADP1 with a 90-bp deletion in *pobA*, affected in 4-hydroxybenzoate hydroxylase	Δ*pobA992*	D. D'Argenio, unpublished data
ADP7529	11.6-kb deletion with endpoints in *dcaE* and the intergenic region between *pcaU* and *pcaI*	Δ*dcaI*	10; this study
ADP8018	0.33-kb *dcaH* deletion created in pZR8044 and introduced into the chromosome of ADP992	Δ*pobA992* Δ*dcaH1*	This study
ADP8023	Km^r; *dcaA*::Km^r from pZR8053 in the chromosome of ADP1	*dcaA1*::Km^r	This study
ADP8061	812-bp deletion in *dcaA* created in pZR8075 and introduced into the chromosome of ADP1	Δ*dcaA2*	This study
ADP8062	100-bp deletion in *dcaE* constructed in pZR8077 and introduced into the chromosome of ADP1	Δ*dcaE2*	This study
Plasmids			
pBBR1MCS	Chl^r; broad-host-range cloning vector		30
pBKS	Ap^r; narrow-host-range cloning vector		Stratagene
pBSK	Ap^r; narrow-host-range cloning vector		Stratagene
pUC4K	Ap^r Km^r; carries antibiotic resistance cassette		Amersham Pharmacia Biotech
pUC19	Ap^r; narrow-host-range cloning vector		46
pZR8000	Chl^r; 34.8-kb *Acinetobacter* sp. strain ADP1 *Sac*I insertion in pBBR1MCS containing *dca* genes as well as *pca, qui,* and *pob* genes		This study
pZR8005	Ap^r; 11.8-kb *Sac*I-to-*Sac*II insertion encompassing *dca* genes from pZR8000 in pBSK		This study
pZR8006	Ap^r; 11.8-kb *Sac*I-to-*Sac*II insertion with *dca* genes from pZR8000 in pBKS; insertion orientation is flipped relative to that of pZR8005		This study
pZR8030	Ap^r; 5.5-kb *Kpn*I deletion of pZR8006		This study
pZR8031	Ap^r; 8.8-kb *Hin*dIII deletion of pZR8006		This study
pZR8038	Ap^r; 8.9-kb *Hin*dIII insertion of pZR8006 in pUC19		This study
pZR8044	Ap^r; 0.34-kb *Bgl*II-*Eco47*III deletion of pZR8031		This study
pZR8053	Ap^r; Km^r cassette from pUC4K in *Xho*I site of pZR8030		This study
pZR8054	Ap^r; 4-kb *Hin*dIII-to-*Eco*RI insertion of pZR8038 in pUC19		This study
pZR8061	Ap^r; 1.68-kb *Hin*dIII-*Sph*I insertion of pZR8006 in pUC19		This study
pZR8075	812-bp *Hin*cII-*Nru*I deletion of pZR8054, which removes much of the *dcaA* gene		This study
pZR8077	100-bp *Msc*I-*Bsg*I deletion of pZR8061, which removes a central piece of the *dcaE* gene		This study

of the vector antibiotic resistance marker. Because Klenow fragment was used to construct the deletions of pZR8044 (in *dcaH*) and pZR8077 (in *dcaE*), with the potential for unintended removal of exposed single-stranded DNA, the deletions of pZR8044 and pZR8077 were sequenced to verify their specificities to those genes.

Insertion or deletion mutations in the *Acinetobacter* chromosome were analyzed by PCR (Fig. 3). In addition, verification that an introduced, defined mutation was responsible for an adipate-negative phenotype was tested by transformation of a mutant strain with a subclone containing the wild-type gene (Fig. 2). ADP8018 (Δ*dcaH1*) was transformed to the adipate-positive phenotype by pZR8031 but not by pZR8054; ADP8061 (Δ*dcaA2*) and ADP8023 (*dcaA1*::Km^r) were corrected to the adipate-positive phenotype by pZR8054 but not by pZR8031; and ADP8062 (Δ*dcaE*2) was made adipate positive by pZR8061 but not by pZR8031 (Fig. 2 and 3).

PCRs. For shorter PCR products, preparation of template DNA followed the instructions provided with InstaGene Matrix from Bio-Rad; longer products were produced using crude chromosomal preparations as the template. The usual PCR conditions were 94°C for 3 min followed by 30 cycles of denaturing at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for an appropriate length of time. Primer sets were as follows (Fig. 3): MGZR80T31 and MGZR80F15 for analysis of Δ*dcaH1* in ADP8018; MGZR80T36 and MGZR80F9 for Δ*dcaE2* in ADP8062; MGZR80T37 and MGZR80F9 for *dcaA1*::Km^r in ADP8023; and MGZR80T38 and MGZR80F7 for Δ*dcaA2* in ADP8061. The oligonucleotide sequences of these primers are as follows: MGZR80F7, 5′-CCT GCT TGT AAT CCA GTG AGA TGA-3′; MGZR80F9, 5′-CAA TAA TTT CTC CAC TGG CAT AAC G-3′; MGZR80F15, 5′-GAA AAT GTC ACA GAT ATT AGT GAA ATT GAC-3′; MGZR80T31, 5′-CGG TAC AGT CAC AGG CAA ACC TG-3′; MGZR80T36, 5′-AGC TGC CAT CAT TTC TTC CTA GAT A-3′; MGZR80T37, 5′-TTA CGG GCT TGG GAC ATT GTG-3′; and MGZR80T38, 5′-ATA GTA GAT TGC TAT AGC GAA ATA TAG AGA-3′.

The primer pair I8pcaU1 (5′-GAT AAC TCC AAT GTG CAT CTA GC-3′) and MGZR80T34 (5′-GCT GCA TCT GCG TAT TCA GTC GT-3′) was used to amplify DNA covering the *dca-pca* deletion of strain ADP7529 by PCR.

DNA sequencing. The *Acinetobacter* DNA insertion of pZR8005 (Fig. 1) was sequenced by primer walking at the Yale Keck Biotechnology Resource Laboratory. Standard ABI PRISM terminator cycle sequencing with AmpliTaq DNA polymerase was used.

Nucleotide sequence accession number. The DNA sequence for the *dca* genes from *Acinetobacter* sp. strain ADP1 may be found under accession no. LO5770 in the GenBank database.

RESULTS

Phenotype of *E. coli* carrying pZR8000. The 33.8-kb *Sac*I insertion of pZR8000 contains *pca, qui*, and *pob* genes as well as putative adipate dissimilation genes (Fig. 1). pZR8000 had a low yield on isolation, probably due to the size of its insertion and the low copy number of the vector. Low copy number likely contributed to its stability in *E. coli*. When tested on gradient plates, *E. coli* DH5α(pZR8000) did not show enhanced growth in the presence of protocatechuate, 4-hydroxybenzoate, quinate, or adipate. Its inability to utilize adipate was not surprising given that it lacked at least one necessary *dca* gene, as discussed below.

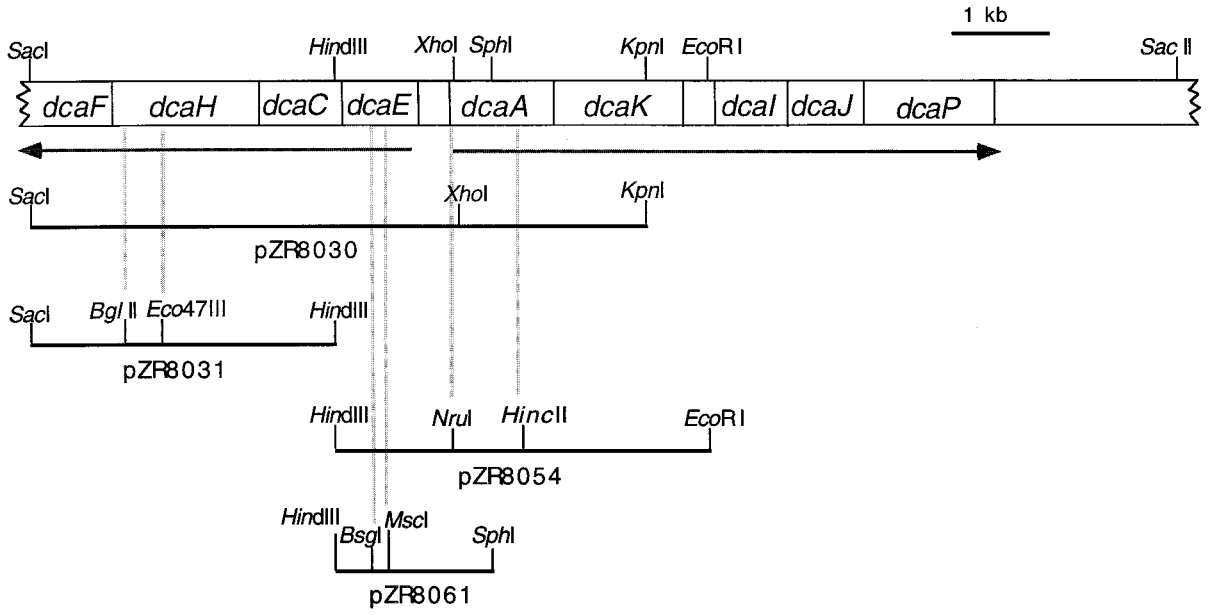

FIG. 2. Subclones used to create mutant strains and to verify their genotypes. The physical map of the *dca* genes shows the location of restriction sites used to create the subclones. Subclones used to create deletion or insertion mutations have additional, internal restriction sites. The gray lines show the sites' locations within the gene that was altered.

A mutant strain of *E. coli* DH5α(pZR8000), designated EDP1(pZR8000′), grew with 4-hydroxybenzoate. To determine if the mutation conferring this phenotype was in the plasmid or in the bacterium, growth properties of the two bacterial strains (DH5α and EDP1) and two plasmids (pZR8000 and pZR8000′) in different combinations were examined. As shown in Table 2, either plasmid conferred upon strain EDP1 the ability to grow with 4-hydroxybenozate, whereas neither plasmid allowed growth of strain DH5α with the compound. Therefore, it is evident that a mutation giving rise to strain EDP1 allowed the strain to use genes in either plasmid to support growth with 4-hydroxybenzoate. Such strains also grew with protocatechuate but not with quinate.

Sequence analysis of the new chromosomal region captured in pZR8000. The G+C content of the 33.8-kb *Sac*I insertion of pZR8000 was 40.9%, typical of strain ADP1. Translation of the DNA sequence of its subclone pZR8005 (Fig. 1) in all six possible reading frames revealed the open reading frames shown in Fig. 3. Note that the *Sac*I end of the pZR8000 clone truncated *dcaF* 516 nucleotides short of its 3′ end (D. Parke, unpublished data); however, the complete sequence of the gene was used in the tables and discussion below.

For each protein encoded by the *dca* region, the highest-scoring homolog revealed by a BLAST search (1) of the nonredundant NCBI database is listed in Table 3. Data from sequence analysis and Table 3 form the basis of the physical map of the *dca* genes shown in Fig. 3. That *dcaECHF* and *dcaAK* form two divergent transcripts is unambiguous. What is less certain, given the distance between *dcaK* and *dcaI*, is whether the genetic unit *dcaIJP* is part of a *dcaAKIJP* transcript or is subject to independent control. At least four of the *dca* genes encoding the products listed in Table 3 have homologs in adjacent regions of the ADP1 chromosome. The relationships between these genes and their products are shown in Table 4.

Most of the deduced protein sequences possess amino acid sequence motifs characteristic of the functional family to which each belongs (Table 5) (25). DcaK, being closely related to MucK, is a member of the aromatic acid transporter subgroup within the major facilitator superfamily (34). The prediction of 12 transmembrane helices in DcaK is in accord with the number predicted for other members of this group of transporters. Although DcaC shares a relatively low level of identity with its closest homolog in the database compared to the other enzymes encoded by the *dca* region (Table 3), it preserves the short-chain dehydrogenase/reductase family signature (Table 5).

Two *dca* gene products that did not register a motif were DcaI and DcaH. The region from residues 18 to 33 of DcaI corresponds to the CoA transferase 1 signature with the exception of two amino acid changes. At residue 24 of DcaI, Thr is present rather than one of the motif amino acids (Leu, Ile, Val, Met, Phe, or Ala), and position 25 has Ser rather than Gly. Analogously, the peptide in DcaH that correlates to the 3-hydroxyacyl-CoA dehydrogenase signature region of the protein, from residues 184 to 209, has several differences from the conserved motif: His192 rather than Arg, Gly198 rather than a motif amino acid (Leu, Ile, Val, Met, Phe, or Tyr), and Asn at 209 rather than the Gly or Val found in the motif sequence. Since some other members of the hydroxyacyl-CoA dehydrogenase family in the NCBI database also fail to elicit the Prosite motif, it is likely that the signature sequence is based on too narrow a database.

DcaP elicited no convincing homologs from a database search (Table 3). The weakness of the match with ScrY, a sucrose-specific porin, is reflected in an alignment restricted to 185 residues at the N termini of the proteins. Moreover, the DcaP and ScrY match had an Expect value of 0.34, which underlines the weakness of their relationship compared with the low Expect values obtained with the other homolog pairs shown in Table 3. When ScrY is aligned with its closest homolog, the maltoporin LamB, the overall identity is only 20%;

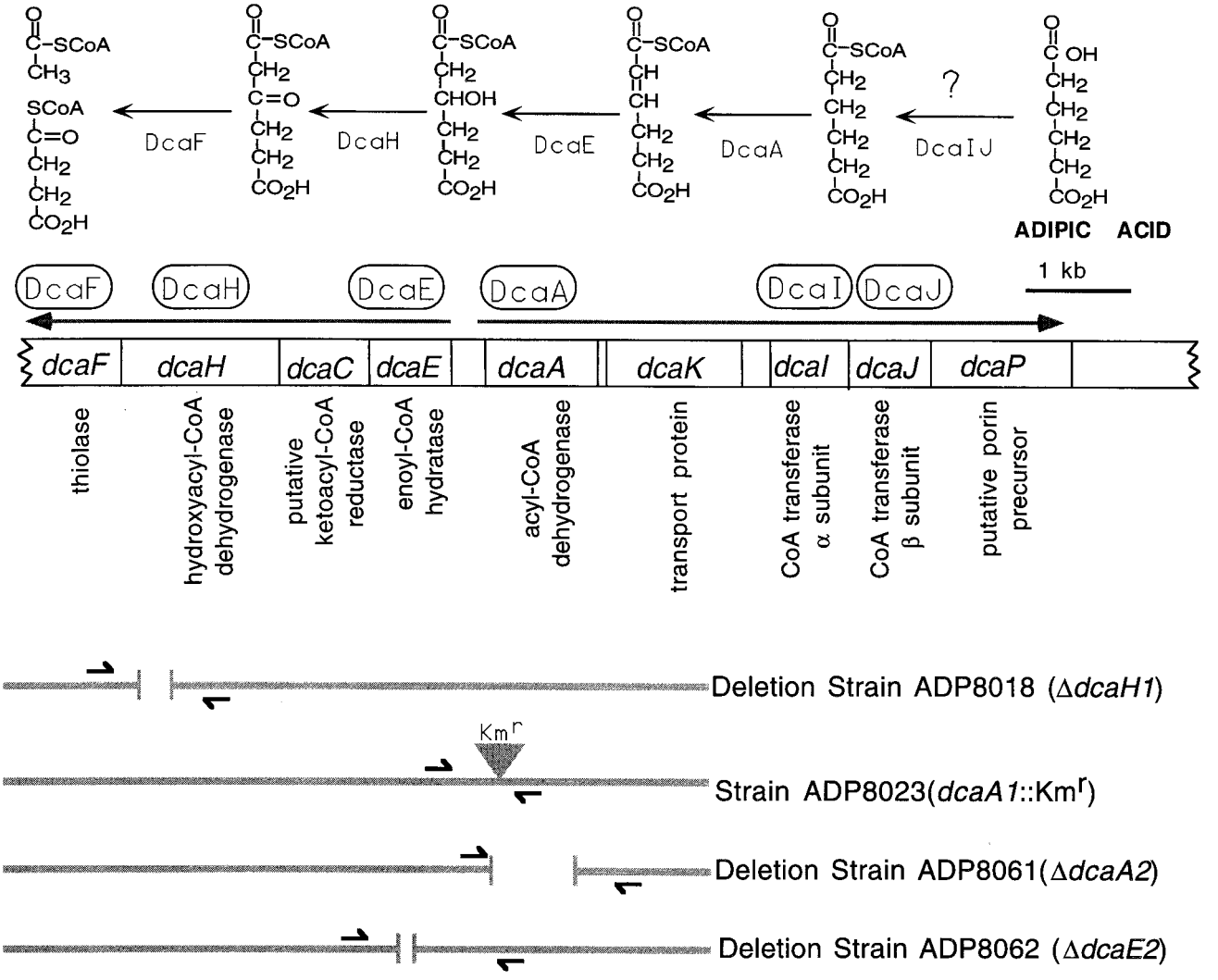

FIG. 3. Proposed roles of *dca* genes and physical maps of *dca* mutations in *Acinetobacter* strains. The *dcaP* gene is proximal to the *pca* genes shown in Fig. 1. Beneath each gene is the likely function of its product, deduced from data presented in Tables 3 and 5. A putative catabolic pathway for adipic acid, which enlists the circled gene products, is shown at the top. Activation of adipic acid is marked by a question mark because it is possible that an as-yet-unidentified ligase carries out the reaction. Small arrows flanking the sites of mutations, aligned beneath the genetic map, denote the locations of primers used to verify mutations.

however, their relationship is revealed convincingly in their similar structures (15, 40). Circumstantial evidence, which includes the prediction that DcaP has a leader sequence (33) (Table 5), suggests that DcaP may be a porin precursor.

Sequence of the *dca-pca* deletion of strain ADP7529. The nucleotide sequence of ADP7529, the recipient used to identify the *E. coli* clone carrying pZR8000, revealed that the strain contains a deletion of 11.6 kb with endpoints in the *dcaE* gene and in the *pcaU-pcaI* intergenic region (Fig. 4).

Adipic acid phenotypes of *Acinetobacter dcaH*, *dcaE*, and *dcaA* mutants. The ability of *Acinetobacter* sp. strain ADP1 to undergo natural transformation allowed introduction of defined deletions and of a Km^r marker into genes located in divergently transcribed regions (Fig. 3). Sequence analysis indicated that the large *dcaA* deletion in ADP8061 should cause a frameshift resulting in only an 18-residue peptide being produced rather than the wild-type protein. DcaE is ordinarily a 262-amino-acid polypeptide; the ADP8062 mutant retains the

TABLE 2. Growth phenotypes of *E. coli* strains with respect to 4-hydroxybenzoate

E. coli strain and plasmid	Growth at the expense of 4-hydroxybenzoate[a]
DH5α	−
DH5α(pZR8000)	−
DH5α(pZR8000′)[b]	−
EDP1	−
EDP1(pZR8000)	+
EDP1(pZR8000′)	+

[a] Cells were grown in minimal medium supplemented with 0.1% yeast extract and chloramphenicol to maintain plasmids. Tubes with 5 mM sodium 4-hydroxybenzoate were compared to those containing no additional carbon source as well as those with 5 mM sodium chloride.

[b] pZR8000′ is the plasmid isolated from mutant EDP1(pZR8000), which grows at the expense of 4-hydroxybenzoate.

TABLE 3. Dicarboxylic acid pathway genes and their products and relationships to other proteins[a]

Gene	Size of gene product (aa)[b]	Related gene products				
		Function (source)	GenBank accession no.	Size of gene product (aa)	% Identity[c]	% Similarity[d]
dcaF	401	Probable acyl-CoA thiolase (*Pseudomonas aeruginosa* PAO1)	AAG06977	401	67	82
dcaH	505	Probable 3-hydroxyacyl-CoA DH[e] (*Pseudomonas aeruginosa* PAO1)	AAG05017	509	52	66
dcaC	253	Ketoreductase (*Streptomyces fradiae*)	S54815	261	34	51
dcaE	262	Enoyl-CoA dehydratase (*Acinetobacter* sp. strain SE19)	AAG10018	258	61	80
dcaA	384	Acyl-CoA DH (*Acinetobacter* sp. strain SE19)	AAG10019	384	82	89
dcaK	435	*cis,cis*-Muconate transport (*Acinetobacter* sp. strain ADP1)	AAC27117	413	55	72
dcaI	223	β-Ka[f] succinyl-CoA transferase, α chain (*Pseudomonas putida*)	AAA25922	231	65	74
dcaJ	224	β-Ka succinyl-CoA transferase, β chain (*Acinetobacter* sp. strain ADP1)	AAC46433	217	69	81
dcaP[g]	438	Sucrose porin precursor (plasmid pUR400, *Salmonella* and *E. coli*)	AAA98417	505	25	39

[a] Based on BLAST program (1) using sequences in the nonredundant protein databases.
[b] aa, number of amino acids.
[c] Percentage of amino acids that are identical between the Dca protein and its aligned homolog.
[d] Percentage of amino acids that are identical or conserved between the Dca protein and its aligned homolog.
[e] DH, dehydrogenase.
[f] β-Ka, β-ketoadipate.
[g] The 3′ end of *dcaP* was sequenced previously (M. Lee and D. Young, unpublished data).

first 93 residues of the wild-type protein, but the mutant protein is presumed to be only 100 residues long due to a frameshift caused by the deletion. The *dcaH* mutation of ADP8018 results in retention of the original reading frame but loss of a 111-amino-acid segment of the translated product.

Note that the Km^r insertion marker used in ADP8023 has a G+C content higher than that typical of most *Acinetobacter* genes. Such a disparity may cause polar effects. This possibility in ADP8023 led to the creation of *dcaA2* deletion strain ADP8061.

Mutant strains ADP8018 (Δ*dcaH1*), ADP8023 (*dcaA1*::Km^r), ADP8061 (Δ*dcaA2*), and ADP8062 (Δ*dcaE2*) grew with doubling times similar to that of the parental strain when the carbon source was succinate, but all failed to grow on adipate (Fig. 5). The phenotype of ADP8062 (Δ*dcaE2*) appeared to be slightly leaky on adipate plates; the effect was heat sensitive, being minimal at 37°C. As expected, the phenotype of ADP992, the parental strain of ADP8018 (Table 1), was similar to that of ADP1 in response to adipate and other dicarboxylic acids provided on gradient plates.

Selection of adipate-positive second-site suppressor mutations. Since ADP8018 (Δ*dcaH1*), ADP8061 (Δ*dcaA2*), and ADP8062 (Δ*dcaE2*) each contained a significant *dca* deletion mutation that eliminated the ability to grow at the expense of adipate, their adipate-negative phenotypes were expected to be very stable. Indeed, the reversion frequency of Δ*dcaH2* mutant strain ADP8018 at 30 or 37°C was below 4×10^{-10}, the limit of detection. By contrast, adipate-positive colonies of ADP8023 (*dcaA1*::Km^r), ADP8061 (Δ*dcaA2*), and ADP8062 (Δ*dcaE2*) did arise on selective plates. When 2×10^8 cells were plated on adipate medium, ADP8061 formed adipate-positive colonies at a frequency of 10^{-7} or lower after 7 days at 30°C. For ADP8023, under similar conditions, the frequency was 3×10^{-7}. The frequency of adipate-positive colonies of ADP8062 at 37°C was 4×10^{-7} or less.

A reduced growth rate on adipate differentiated the adipate-positive mutant derivatives from ADP1 and indicated that they were not true revertants. Strains carrying putative second-site suppressor mutations were purified, and chromosomal lysates of the strains were prepared. Purified, representative adipate-

TABLE 4. Dicarboxylic acid pathway genes and their homologs in flanking regions of the ADP1 chromosome

dca gene of homolog(s)	% G+C content		% nt[a] identity	Distance (kb) between *dca* gene and homolog	Function(s) of gene product	% aa[b] identity (% similarity)	GenBank accession no. or reference
	dca gene	Homolog					
dcaI	40				Putative Dca[c] succinyl-CoA transferase, α chain		This study
pcaI		55	61	8.4	β-Ketoadipate succinyl-CoA transferase, α chain	61 (75)	AAC46432
dcaJ	41				Putative Dca adipate succinyl-CoA transferase, β chain		This study
pcaJ		54	65	8.4	β-Ketoadipate succinyl-CoA transferase, β chain	68 (80)	AAC37147
dcaF	44				Putative Dca-CoA thiolase		This study
pcaF		56	59	16	β-Ketoadipyl-CoA thiolase	56 (70)	AAC37148
dcaK	41				Putative Dca transport		This study
mucK		40	63	12[d]	*cis,cis*-Muconate transport; possible adipate transport	55 (72)	AAC27117
pcaK		42.5	Divergent	13.5	Protocatechuate transport	21 (37)	AAC37151

[a] nt, nucleotide.
[b] aa, amino acid.
[c] Dca, dicarboxylic acid.
[d] Based on sequence analysis of DNA between *mucK* and *dcaF* (D. Parke, unpublished data).

TABLE 5. Motifs conserved in *dca* gene products[a]

Gene product	Motif peptide in Dca protein	Location in protein[b]	Family for which motif is characteristic
DcaF	VNRLCASGLAAIIDSARAI	85–103	Thiolase acyl-enzyme intermediate
	NPNGGAIAVGHPLGASG	346–362	Thiolase signature 2
	AVVSLCIGVGQGLA	381–394	Thiolase active-site motif
DcaC	SIAGRMGYPFRLAYSTSKWGIVGFTKTLS	135–163	Short-chain DH[c]/reductase signature
DcaE	IAAVNGYALGGGCELAMHTDI	103–123	Enoyl-CoA hydratase/isomerase signature
DcaA	CLTEPEAGSDAAS	123–135	Acyl-CoA DH signature 1
	QIHGGAGYISEYSIERFYRD	337–356	Acyl-CoA DH signature 2
DcaK	AGMIADKLGRRAVFAFGT	304–321	Sugar transport protein signature 1
	FFGFLYGIPYAINATYMTESFPTSIR	346–371	Sugar transport protein signature 2
DcaJ	LHSENGVLA	47–55	CoA transferase signature 2
DcaP precursor	MKKLILAVACATASGTLLA	1–19	Signal peptide motif

[a] Based on patterns stored in the Prosite database (25), except for the signal peptide motif (33).
[b] Position of motif amino acid residues in the *dca* protein.
[c] DH, dehydrogenase.

positive derivatives of ADP8061 and ADP8062 were analyzed by PCR and were shown to have retained their original deletions.

Because ADP8023 had an easily monitored Km^r marker, its phenotypic revertants received the greatest attention. Their phenotypes with respect to growth rate on adipate varied, and 36% of them were temperature sensitive, failing to grow on adipate at 37°C. All of them carried the Km^r marker. Moreover, when the Km^r marker of representative phenotypic revertant strains was transformed into ADP1, it conferred an adipate-negative phenotype. In addition, DNA from the ADP8023 suppressor mutants was able to transform two mutant strains to an adipate-positive phenotype: ADP8023 as well as ADP8061, which contains a deletion that flanks the Km^r marker in ADP8023 (Fig. 3). One class of ADP8023 suppressor mutants proved to be unstable, throwing off adipate-negative colonies on subculture; its phenotype was interpreted to be caused by unstable amplification of a genetic region encoding low-level suppressor activity.

Utilization of fatty acids, pimelic acid, and longer-chain dicarboxylic acids. A gradient plate method was used to assess the ability of mutant strains to utilize medium-chain fatty acids. The fatty acids caprylic and capric acid (8 and 10 carbons, respectively) were metabolized by all, indicating that the *dca* genes under investigation are not involved in ω-oxidation of these particular fatty acids.

Strain ADP1 and the three deletion strains were also screened for their ability to utilize longer-chain dicarboxylic acids. Figure 5 shows that ADP1 grew at the expense of all of the tested compounds. The doubling time of adipate-grown cultures of ADP1 at 37°C was about 30 min. The doubling time of dodecanedioic acid- or tetradecanedioic acid-grown cultures was 2.6 or 4.7 times longer, respectively. ADP1 cells were very slow to grow at the expense of pimelic acid; as noted in the legend to Fig. 5, an extra day was allowed for growth of cells at the expense of pimelic acid as well as tetradecanedioic acid.

At a carbon chain length of 14, solubility of the saturated dicarboxylic acids in minimal medium starts to fall off, and at 16 atoms, insolubility obscures differences in the amount of growth. In the case of hexadecanedioic acid, the amount of growth was determined by viable cell counts on minimal medium plates containing succinate. Under the conditions described in the legend to Fig. 5, an ADP1 culture yielded 10^8 cells ml^{-1} on hexadecanedioic acid compared to the DMSO control of 7×10^6 cells ml^{-1}. Although the same number of carbon atoms was provided to cultures with each substrate, decreasing solubility and the tendency of cells to clump onto

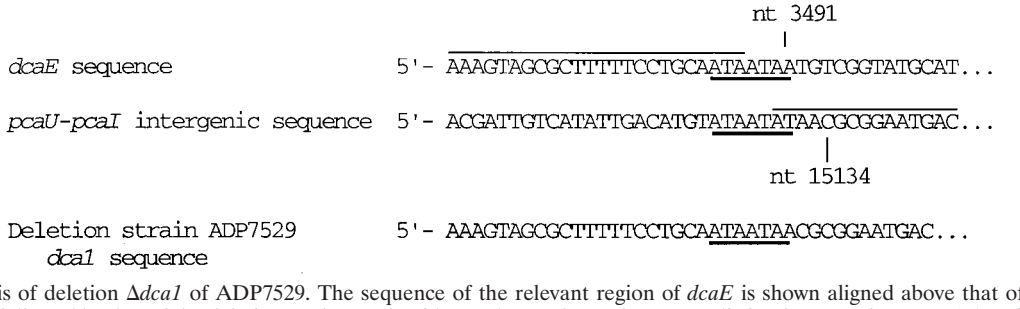

FIG. 4. Analysis of deletion Δ*dca1* of ADP7529. The sequence of the relevant region of *dcaE* is shown aligned above that of the *pcaU-pcaI* intergenic region, followed by that of the deletion strain. Nucleotide numbers refer to the current listing for accession no. LO5770 in the GenBank database. The repeated element present in all three sequences is underlined. Overlining designates hypothetical parent sequences that may have contributed to the deletion juncture; they were arbitrarily chosen to clarify the subtle changes at the juncture.

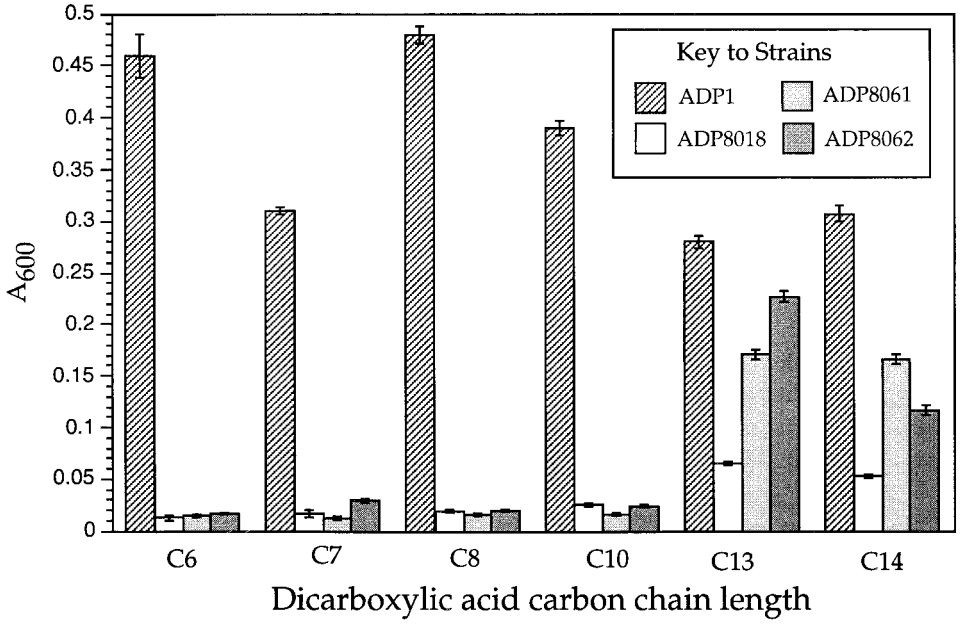

FIG. 5. Relative yields of wild-type and *dca* mutant *Acinetobacter* strains grown at the expense of dicarboxylic acids of different chain length. Strains represented in the legend are ADP1, wild type; ADP8018, Δ*dcaH1*; ADP8062, Δ*dcaE2*; and ADP8061, Δ*dcaA2*. Dicarboxylic acids of different chain length are C_6, adipic acid; C_7, pimelic acid; C_8, suberic acid; C_{10}, sebacic acid; C_{13}, tridecanedioic acid; and C_{14}, tetradecanedioic acid. Adipic acid was supplied at 2 mM, and other dicarboxylic acids were supplied at a concentration that provided an equivalent molarity of carbon atoms. The optical density of a mutant culture on each substrate was measured at the time that wild-type cells, grown in parallel on the same substrate, reached a plateau in density. For two of the dicarboxylic acids (chain length C_7 and C_{14}), this required an extra overnight period of growth.

insoluble crystals may account for the apparent reduced growth of ADP1 on the longer-chain acids shown in Fig. 5.

When cultured on adipic, pimelic, suberic, or sebacic acid, strains mutated in *dcaE*, *dcaA*, and *dcaH* failed to grow (Fig. 5). Strain ADP8023 has a similar phenotype on these substrates (data not shown). Clearly, the *dcaA, dcaE*, and *dcaH* gene products are specific for at least the medium-chain dicarboxylic acid thioesters. Growth at the expense of homologs longer than sebacic acid was reduced in the mutant strains compared to those of ADP1 but to a lesser extent (Fig. 5). Viable counts from tetradecanedioic acid cultures confirmed that the optical density at 600 nm reflected the number of cells present. Finally, growth at the expense of hexadecanedioic acid yielded about 27% of the viable count of ADP1 for ADP8061 and ADP8062; it was 16% of the wild-type level in ADP8018 (data not shown).

Reversion of mutant strains can be ruled out as an explanation for the disproportionately high relative yields of ADP8061 and ADP8062 on the longer-chain dibasic acids. After exposure to tridecanedioic or tetradecanedioic acid, cultures of ADP8061 or ADP8062, which showed an elevated amount of growth, continued to show the same adipate-negative phenotype as the original strains. Moreover, the relatively elevated amount of growth of the two strains on the higher-carbon-number homologs versus that on adipate was observed on solidified medium containing tetradecanedioic acid as the sole carbon source, where suppressor mutants or revertants could be observed directly.

Ability of mutant strains to dissimilate glutarate. Dissimilation of even- and odd-chain dicarboxylic acids is postulated to diverge at the shorter thioesters: even-numbered compounds would be converted to succinyl-CoA following β-oxidation steps (Fig. 3), whereas odd-numbered ones would be converted to the C_5 derivative glutaryl-CoA. Indeed, in another strain of *Acinetobacter*, glutarate was found to accumulate when chloramphenicol-inhibited cells were incubated with pimelic acid or its C_9, C_{11}, or C_{13} homologs (8). Evidence for metabolism of pimelate via a glutaryl-CoA intermediate under anaerobic conditions also exists (16, 20).

Although strain ADP1 may well metabolize odd-chain-length dicarboxylic acids through glutaryl-CoA, it does not grow with externally supplied glutarate alone. However, growth with external glutarate is observed if 0.1 mM adipate is added to the medium (D. A. D'Argenio, personal communication). Strain ADP7529 was shown to have a large deletion that extends into the *dcaE* gene (Fig. 1 and 4). Because the strain lacks the intergenic region between the *dcaECHF* transcript and the divergently transcribed *dcaA*, it is unlikely to express *dcaH* as well as *dcaA* and *dcaE*. Although unable to utilize pimelate, ADP7529 maintained its ability to grow at the expense of glutarate in the presence of 0.1 mM adipate (data not shown). This rules out any role for DcaA, DcaE, or DcaH in the metabolism of glutaryl-CoA.

Indeterminate role of other genes identified on pZR8005. The functions of the other open reading frames identified on pZR8005 (Fig. 3 and Table 3) are under investigation. Initial analysis of knockout mutations in several of these genes has revealed that they do not lead to simple adipate-negative phenotypes. Nevertheless, *dcaK* and *dcaC* are located on transcripts which encode genes shown to be required for adipic acid dissimilation. Induction of *dcaP::lacZ*-Km^r by adipate in *Acinetobacter* (D. Parke, unpublished data) demonstrates that

the putative transcript *dcaIJP*, which may be part of a longer *dcaAKIJP* transcript, also encodes genes which participate in dicarboxylic acid metabolism.

DISCUSSION

***E. coli* mutant with improved potential to dissimilate phenolics.** Isolation of a large clone, pZR8000, that contained not only genes that corrected an adipate-negative mutant but also suites of genes involved in aromatic and hydroaromatic catabolism led us to examine the phenotypes of *E. coli* carrying the clone. The full potential of DH5α(pZR8000) to utilize protocatechuate and 4-hydroxybenzoate required a mutation giving rise to *E. coli* strain EDP1 (Table 2). Although the scope of our research was limited to a single, genetically uncharacterized mutant strain, it opens the door to expanding the catabolic potential of *E. coli* as an experimental vehicle. It is not clear why the hydroaromatic compound quinate was not readily catabolized by the mutant *E. coli* harboring genes for all of the requisite enzymes and transporter, but this may be related to the periplasmic conversion of quinate to protocatechuate in *Acinetobacter* sp. strain ADP1 (12).

Analysis of the *dca-pca* deletion of strain ADP7529. It is an indication of the remarkable natural competence of the "Juni" strain of *Acinetobacter* (28) that our cloning efforts were successful in spite of the hurdle of screening clones against a recipient strain that turned out to have a relatively large, 11.6-kb deletion. The sequences of the large deletions found in previous studies of *Acinetobacter* (13, 18) have not been determined, and it is not clear why they arise at high frequency upon application of the Δ*pcaBDK* positive selection method (21). An explanation might be that they are mediated by crossing over between distant copies of a transposon or insertion sequence such as IS*1236*, which occurs seven times in the ADP1 chromosome (17). However, the sequence of Δ*dca1* in ADP7529, isolated in a Δ*pcaBDK* positive selection (10), gives no evidence that IS*1236* played a role in this deletion. Although the molecular events that created the deletion are unknown, it is noteworthy that the sequence ATAATA, itself a direct repetition, which is separated by 11.6 kb, occurs at the deletion juncture (Fig. 4).

Phenotypic evidence for overlapping functions of β-oxidation genes in *Acinetobacter*. Data based on sequence analysis and mutant phenotypes support the conclusion that DcaA, DcaE, and DcaH act on thioesters of adipic, pimelic, and suberic acids as depicted in Fig. 3. Growth studies and analysis of pseudorevertants suggest that homologs of the cloned *dca* genes exist in strain ADP1. It remains to be determined whether such homologs are required for the conversion of longer-chain thioesters to medium-chain ones, which are then subject to catalysis by DcaA, DcaE, and DcaH. Results with the Δ*dcaH1* mutant ADP8018 are consistent with this hypothesis, showing an elevated growth yield on the longer dibasic acids that would be predicted by liberation of only one or two acetyl units per chain (Fig. 5). It is difficult to explain the elevated growth yields of ADP8061 and ADP8062 at the expense of tridecanedioic or tetradecanedioic acid. It is possible that a putative DcaA or DcaH homolog that acts preferentially on longer-chain acids may possess some activity towards certain shorter-chain derivatives, which could be enhanced by amplification of the coding segment of the chromosome. Although the physiological data do not allow us to determine the absolute limits of specificity for the three cloned *dca* gene products, they do indicate a role for them in at least part of the dissimilation of the longer-chain acids.

It is also possible that other enzymes, not directly engaged in straight-chain dicarboxylic acid dissimilation, may act nonspecifically on the longer-chain acids. Isolation of an array of phenotypic revertants of *dcaA* and *dcaE* mutants provides genetic evidence for the presence of related β-oxidation genes in the *Acinetobacter* chromosome. The inference that other genes with functions similar to those of *dcaE* or *dcaA* are present is not surprising given the array of DcaA, DcaE, and DcaH homologs from single bacterial strains pulled up in database queries. Further characterization of the suppressor mutant strains and the *dca* genetic region afford synergistic lines of investigation.

Interpretation of functions of other open reading frames in the *dca* region of pZR8000. In addition to the three open reading frames that were the focus of this study, six other genes were identified as part of the *dca* region on pZR8000. The *dcaF* gene, encoding a thiolase homolog, is presumed to be required for thiolytic cleavage of acetyl-CoA from acyl-CoA. The role of the *dcaC* gene is unknown. The products of *dcaK* and *dcaP* are being investigated for their roles in the control of dicarboxylic acid traffic into the cell. Genetic linkage of *dcaP* with *dcaIJ* is seen as a tantalizing hint of the functional relationship of these genes' products. The *dcaIJ* genes are located not far from the homologous *pcaIJ* genes, which encode a β-ketoadipate succinyl-CoA transferase involved in protocatechuate catabolism (Table 4). The *dcaIJ* genes undoubtedly encode the third enzyme (in addition to PcaIJ and CatIJ) with β-ketoadipate succinyl-CoA transferase activity previously detected in *Acinetobacter*; this broader-specificity enzyme was induced by adipate, and unlike PcaIJ and CatIJ, its activity was inhibited by adipate (7). Activity of this presumed adipate succinyl-CoA transferase towards longer dicarboxylic acids remains a subject for future investigation. In *P. fluorescens*, an adipate succinyl-CoA transferase did not appear to act on dicarboxylic acids longer than pimelate; a separate ligase was responsible for activation of longer-chain acids (23, 24). If this is the case in *Acinetobacter*, a critical ligase gene remains to be discovered.

Relationships of *dca* gene products to homologous *Acinetobacter* proteins. The closest homologs of DcaE and DcaA elicited by BLAST queries are those from *Acinetobacter* sp. strain SE19 (Table 3). The homologous SE19 genes, termed *fadB* and *fadE*, respectively, lie next to a cluster of genes required for the conversion of cyclohexanol to adipic acid (9). Only 147 residues of the deduced N-terminal sequence of FadC, the DcaH homolog, from strain SE19 have been deposited in GenBank, and therefore this enzyme was not pulled up in a database query; however, alignment of the N-terminal sequences of FadC and DcaH demonstrated a sequence identity of 55%. Organization of the three *fad* genes from strain SE19 is similar to that of the *dca* homologs, with the exception of the intervening *dcaC* in ADP1. Given their location adjacent to genes required for generating adipic acid from cyclohexanol and their close relationship to the *dca* homologs, the SE19 *fad* genes probably encode enzymes for adipic acid dissimilation.

In *Acinetobacter* sp. strain ADP1, the region between *dcaK* and *mucK* spans 12 kb of DNA which encodes additional genes involved in dicarboxylic acid metabolism (D. Parke and G. Peterson, unpublished data). The location of and close relationship between DcaK and MucK (Tables 3 and 4) suggest that phenotypic masking by the DcaK transporter may have obscured adipic or other dicarboxylic acid carrier functions of the *cis,cis*-muconate transporter in a *mucK* mutant (44).

Organization of putative *dca* genes in *Pseudomonas aeruginosa*. Like *Acinetobacter* sp. strain ADP1, *P. aeruginosa* strains are able to grow at the expense of dicarboxylic acids containing from 6 to 10 carbon atoms (42). As mentioned above, early work established the presence of two genes that contributed to activating dicarboxylic acids in the closely related *P. fluorescens* (23, 24). In light of that work and the identification of *P. aeruginosa* homologs of the Dca proteins from strain ADP1 (Table 3), the *P. aeruginosa* PAO1 genome (43) was examined to determine the organization and location of putative *dca* gene homologs.

One likely *dca* genetic region in the *P. aeruginosa* PAO1 genome lies at position 1771122 to 1775757. Giving genes the *dca* designations, a putative *dcaEH* transcript is divergently transcribed from a likely *dcaRA* transcript. Identification numbers for the PAO1 genes are indicated in parentheses: *dcaE* (PA1629), *dcaH* (PA1628), *dcaR* (PA1630), and *dcaA* (PA1631). In *Acinetobacter, dcaR* is linked to *dcaF* and has been identified tentatively as one of the regulatory genes for dicarboxylic acid dissimilation (D. Parke and G. Peterson, unpublished data). In *P. aeruginosa*, this gene cluster lies between sets of genes associated with transport. As discussed below, it seems likely that they play a role in medium-chain dicarboxylic acid dissimilation.

A second cluster of putative *dca* genes, at position 4021918 to 4028538 in the PAO1 genome, forms a unit of transcription that includes *dcaA*, a gene of unknown function, *dcaE, dcaH, dcaF*, and a probable porin gene. These genes have gene identification numbers as indicated parenthetically: *dcaA* homolog (PA3593), *dcaE* homolog (PA3591), *dcaH* homolog (PA3590), *dcaF* homolog (PA3589), and the possible porin gene (PA3588).

In all cases, the DcaE, DcaH, and DcaA homologs which correspond to the genes of the first cluster at position 1771122 to 1775757 are more similar to the ADP1 proteins deduced in this study than are those in the second cluster. The fact that the *dcaF* gene product is very similar to that of the ADP1 homolog (Table 3) is taken as evidence supporting the hypothesis that the second cluster is related to dicarboxylic acid degradation as well.

The two PAO1 DcaH homologs bear a close similarity to each other, as do the two DcaE homologs; the aligned proteins possess amino acid identities of 66 and 72%, respectively. By contrast, the two PAO1 DcaA homologs are quite divergent, sharing only 29% amino acid identity in the aligned portion of the proteins. Only DcaA homolog PA1631 is very similar to its ADP1 homolog. In addition, the DcaA homolog PA3593 is 191 amino acids longer than DcaA homolog PA1631. The size difference suggests that the longer protein may be adapted for longer-chain substrates, as occurs in fatty acid degradation. The genes in the region of position 4021918 to 4028538 of the PAO1 chromosome may have evolved to play a role in long-chain dicarboxylic acid dissimilation, and those in the region of position 1771122 to 1775757 may be adapted to shorter compounds such as adipate. Curiously, there is no close homolog for *dcaIJ* in the PAO1 genome, and different transporter genes appear to be associated with the putative *dca* genes.

Phylogenetic trees based on rRNA sequences place *Acinetobacter* and *Pseudomonas* species on neighboring twigs of the same branch (45). The common elements in dicarboxylic acid degradation appear to form a mere skeleton, which was fleshed out by appropriating distinct genes in new contexts.

Genomic context of *dca* genes and intimations of host bacterial niche. In *Acinetobacter* sp. strain ADP1, linkage of genes for associated functions is a striking feature of the *pca-qui-pob-ppa* region (Fig. 1). Proximity of the *dca* genes to the *pca* region raises the question of whether the *dca-pca* linkage provides a selective benefit. In addition to allowing horizontal transfer of a suite of genes, linkage enables them to be amplified (10, 38) when the concentrations of their specific, related substrates are low. Suberin is a complex, natural polyester that could potentially yield substrates for *dca, pca*, and *ppa* gene products. Synthesized by plants as a protective barrier in response to wounding, its components include hydroxycinnamic acids esterified to dicarboxylic acids (5, 19, 32). Typically, dicarboxylic acids found in suberin networks are comprised of at least 16 carbon atoms. As we have demonstrated in this paper, *Acinetobacter* sp. strain ADP1 is capable of degrading the long-chain hexadecanedioic acid. Given these elements, it is tempting to term the *dca-pca-qui-pob-ppa* region of the ADP1 genome a "suberon."

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