Genes for Chlorogenate and Hydroxycinnamate Catabolism (hca) Are Linked to Functionally Related Genes in the dca-pca-qui-pob-hca Chromosomal Cluster of Acinetobacter sp. Strain ADP1

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Hydroxycinnamates are ubiquitous in the environment because of their contributions to the structure and defense mechanisms of plants. Additional plant products, many of which are formed in response to stress, support the growth of Acinetobacter sp. strain ADP1 through pathways encoded by genes in the dca-pca-qui-pob chromosomal cluster. In an appropriate genetic background, it was possible to select for an Acinetobacter strain that had lost the ability to grow with caffeate, a commonly occurring hydroxycinnaminate. The newly identified mutation was shown to be a deletion in a gene designated hcaC and encoding a ligase required for conversion of commonly occurring hydroxycinnamates (caffeate, ferulate, coumarate, and 3,4-dihydroxyphenylpropionate) to thioesters. Linkage analysis showed that hcaC is linked to pobA. Downstream from hcaC and transcribed in the direction opposite the direction of pobA transcription are open reading frames designated hcaDEFG. Functions of these genes were inferred from sequence comparisons and from the properties of knockout mutants. HcaD corresponded to an acyl coenzyme A (acyl-CoA) dehydrogenase required for conversion of 3,4-dihydroxyphenylpropionyl-CoA to caffeoyl-CoA. HcaE appears to encode a member of a family of outer membrane proteins known as porins. Knockout mutations in hcaE confer no discernible phenotype. Knockout mutations in hcaG indicate that this gene encodes a membrane-associated esterase that hydrolyzes chlorogenate to quinate, which is metabolized in the periplasm, and caffeate, which is metabolized by intracellular enzymes. The chromosomal location of hcaG, between hcaC (required for growth with caffeate) and quiA (required for growth with quinate), provided the essential clue that led to the genetic test of HcaG as the esterase that produces caffeate and quinate from chlorogenate. Thus, in this study, organization within what is now established as the dca-pca-qui-pob-hca chromosomal cluster provided essential information about the function of genes in the environment.

Hydroxycinnamates are abundant in plants and are used in both structural (3, 4, 61) and chemical (6) plant defense strategies. These compounds occur freely or as components of plant polymers, such as suberin (3, 4, 23, 38, 61). Highly significant among the hydroxycinnamates is chlorogenate, which is the most abundant phenolic compound in potato tubers (37) and accounts for 1.5% of the dry weight of defatted sunflower oil (22). Chlorogenate has numerous functions, including effects on disease resistance (39) and on the palatability of leaves to insects (27). The production of animal feeds is hindered by the presence of chlorogenic acid, which binds to proteins and accounts for toxicity of carboxymuconate, the product of protocatechuate 3,4-dioxygenase (54, 55) or Aspergillus japonicus (45, 46). A preparation of the latter enzyme is commercially available (http://www.kikkoman.co.jp/bio/j/rinsyou/enzymes/) and is used to control bitterness and to prevent enzymatic browning in the preparation of juice, wine, and coffee. Chlorogenate esterase has been identified as an intracellular enzyme in gut bacteria (7), but little is known about genes encoding this enzyme or how their expression is controlled.

Here we describe bacterial hca genes for catabolism of the ester chlorogenate and the free hydroxycinnamates caffeate, ferulate, coumarate, and 3,4-dihydroxypropionyl coenzyme A (3,4-dihydroxypropionyl-CoA) (Fig. 1). The genetic analysis was conducted with Acinetobacter sp. strain ADP1 (also known as strain BD413 [31]) because of this organism’s extraordinary competence for natural transformation (30). An additional convenience for study of catabolic genes was afforded by the toxicity of carboxymuconate, the product of protocatechuate 3,4-dioxygenase. As summarized in Fig. 1, strains unable to metabolize carboxymuconate can be used to select mutants defective in protocatechuate 3,4-dioxygenase and in metabolic pathways that converge upon protocatechuate (26). The initial evidence (2) for clustering of Acinetobacter genes for metabolic pathways converging through protocatechuate emerged from characterization of mutant strains blocked in expression of pcaHG, which are structural genes for protocatechuate oxygenase, and pobA, which is the structural gene for p-hydroxy-

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benzoate hydroxylase (Fig. 1). Chromosomal fragments containing pobA and surrounding DNA were cloned (2, 13). Analysis of open reading frames between pcaHG and pobA revealed that the quiBCXA cluster is required for conversion of quinate to protocatechuate (17, 18).

Spontaneous mutants lacking pcaHG often have large deletions (20), and some of these deletions prevent growth with straight-chain dicarboxylic acids (8). This finding led to elucidation of dca genes required for growth with the acids having chain lengths ranging from 6 to 16 carbon atoms (48). The dicarboxylic acids and hydroxycinnamates are major components of suberin (3, 4, 23, 38, 61). Thus, the overall dca-pca-qui-pob cluster was known to contain independently transcribed groups of genes that allow growth with plant products, including dicarboxylic acids (dca), protocatechuate (pca), quinate (qui), and p-hydroxybenzoate (pob). An exception to this pattern of clustering was provided by the unlinked vanAB genes encoding the demethylase that converts vanillate to protocatechuate (56).

Vanillate is an intermediate in catabolism of ferulate (11, 19, 44, 51, 58). The location of Acinetobacter genes for metabolism of ferulate and other hydroxycinnamates was unknown. As described here, a strain containing a spontaneous mutation blocking catabolism of a hydroxycinnamate, caffeate (Fig. 1), was isolated, and a cloned restriction fragment containing pobA was shown to restore wild-type function to the mutant strain. Designed mutations altering DNA within the fragment allowed identification of open reading frames required for metabolism of hydroxycinnamates, including chlorogenate (5-O-cafeoylquinic). The newly discovered genes expand our knowledge of the dca-pca-qui-pob-hca supraoperonic cluster, which makes a major contribution to the metabolic versatility of Acinetobacter. The phenotypic characterization of clustered genes provides clues about biological sources of the mixtures of nutrients used by bacteria in the natural environment.

**MATERIALS AND METHODS**

**Strains and culture conditions.** Plasmids and relevant mutants of Acinetobacter sp. strain ADP1 are listed in Table 1. Acinetobacter strains were routinely grown at 37°C in mineral medium supplemented with 10 mM succinate, 5 mM p-hydroxybenzoate, or one of the following compounds at a concentration of 3 mM: caffeate, quinate, coumarate, 3,4-dihydroxyphenylpropionate, protocatechuate, chlorogenate (5-O-cafeoylquinic), or ferulate. These chemicals were supplied by Sigma Chemical Company. The chemical instability of caffeate, protocatechuate, and chlorogenate demanded that plates containing these carbon sources be prepared within 24 h of use. Experiment cells strain DH5a was obtained as a frozen suspension from Gibco BRL. Escherichia coli strain DH5a was grown in Luria-Bertani (53) broth at 37°C. Growth media were supplemented as required with ampicillin (100 µg/ml) or kanamycin (100 µg/ml).

**DNA manipulation.** General previously described procedures (53) were used for manipulation of DNA. Instructions from Bio-Rad were followed when InstaGene matrix was used to isolate chromosomal DNA and for PCR. PCR mixtures (50 µL) used to amplify DNA for sequencing contained 2 µL of supernatant liquid separated from the InstaGene matrix as a template, 2 U of Pfu DNA polymerase, and 50 nM of each primer. PCR products were separated by electrophoresis through a 0.8% agarose gel.
polynucleotide (Stratagene), 10 pmol of each primer, 10 pmol of each deoxyribo-
side triphosphate, and buffer. Amplification was carried out for 30 cycles under
the following conditions: denaturation at 94°C for 45 s, annealing at 56°C for 45 s,
and elongation at 72°C for 90 s. There was a final extension step consisting of 10
min at 72°C. Plasmid DNA was isolated from E. coli either by alkaline lysis (5)
or with Qiaprep columns (Qiagen).

Cloning of hca genes. Plasmid pZR400 containing pobA and DNA downstream of
this gene was prepared as part of an earlier investigation (2). An XhoI-PvuII
subclone of pZR400 was prepared in pGEM3Z(+) (Promega), and the result-
ning plasmid was designated pZR9500 (Table 1; Fig. 2). Subsequent character-
ization of this plasmid and other plasmids containing Acinetobacter DNA re-
vealed that during preparation of pZR9500 a separate chromosomal fragment
had been ligated into an EcoRI site at 8,220 bp downstream of the XhoI site (D.
Parke, unpublished data). Therefore, the rest of the present investigation was
directed to DNA from the XhoI-EcoRI segment of the chromosome (Fig. 2).

DNA sequencing. Acinetobacter DNA was sequenced from the pZR9500 in-
sert, and PCR products were generated from the Acinetobacter sp. strain ADP1
chromosome under standard conditions. All PCR products were gel purified by
using a QiAquick gel extraction kit (Qiagen) according to the manufacturer’s
instructions. A primer walking strategy was utilized for sequencing the previously
uncharacterized DNA. Sequencing reactions were performed by the Yale Keck
Biotechnology Resource Laboratory.

Engineering of deletion mutants. Donor DNA containing designed mutations
altering hcaG were selected in strain ADP9500 (ΔpobA-hcaG1), which contains
a deletion extending from pobA into hcaG (Fig. 2). Donor fragments contained
the deleted portion of pobA and an engineered mutation in hcaG. Selection for
pobA function yielded recombinants containing the hcaG mutation.

The ΔpobA-hcaG1 deletion was created by removing two MfeI fragments from
pZR9501; these fragments comprised a 1,126-bp region spanning the 3’ end of
pobA and the 3’ end of hcaG (Fig. 2). The resulting plasmid, pZR9507, was used
to transform ADP230 (ΔpcaBΔK1). Transformants were selected by using min-
eral medium plates supplemented with 5 mM p-hydroxybenzoate and 10 mM
succinate (Fig. 1). Colonies arising on these plates were struck for single colonies
on 10 mM succinate. InstaGene-isolated template was used in PCR to confirm that
the deletion plasmid had recombined into the chromosome. The ΔpcaBDK1
deletion was replaced with wild-type DNA from pZR3 (20), and this was fol-
lowed by selection for growth with protocatechuate. A transformant emerging
from this selection was designated strain ADP9506 and was used as a recipient
in further selections.

The deletion mutation ΔhcaG1 was prepared by ligation of amplicons AB and
CD to form ABCD, which was further amplified by PCR (Table 2). All PCR were
carried out in 100-µl mixtures containing a 1:100 dilution of plasmid pZR9500
DNA as the template, each deoxynucleoside triphosphate at a concentration of
200 µM, each primer at a concentration of 1 µM, 5 U of Pfu polymerase
(Stratagene Inc., La Jolla, Calif.), and 1× reaction buffer. The reactions were
performed for 25 cycles consisting of 94°C for 45 s, annealing at 56°C for 60 s, and
extension at 76°C for 90 s, followed by a final extension at 72°C for 9 min.
Following gel purification, 5 µl of each PCR product was phosphorylated at 37°C
for 30 min in a 50-µl reaction mixture containing 1 mM ATP, 50 U of T4
polynucleotide kinase (New England Biolabs), and 1× reaction buffer. Prior to
the addition of polynucleotide kinase, the reactants were heated at 70°C for 5
min. Following phosphorylation, ligation was carried out in a 15-µl mixture
containing 5 µl of each kinase reaction mixture, 1× buffer, and 600 U of T4 DNA
ligase (New England Biolabs) for 15 min at room temperature. The deletion
construct was amplified from 1 µl of the ligation reaction mixture, and all other
parameters were the same as those used to generate the products used for
ligation. PCR products from the reaction in which the ligation mixture was used
as the template were electrophoresed on a 0.8% (wt/vol) agarose gel. A single
band corresponding to the desired construct was purified and used as donor
DNA for a transformation in which ADP9500 was the recipient and selection for
growth with p-hydroxybenzoate was imposed. The identity of the engineered
deletion mutation ΔhcaG1 in transformant strains was confirmed by sequence
analysis, and the growth properties of one of the transformants, strain ADP9501
(Table 1), were examined.

Similar conditions were used to generate strain ADP9503 (ΔhcaF2). Ampli-
cons CD and EF (Table 2) were ligated, and the product was amplified to form
the PCR product CDEF containing ΔhcaF2. This DNA was used to transform
strain ADP9502 (hcaF1::Km'), and the resulting population was screened for
kanamycin-sensitive strains. The designed ΔhcaF2 deletion in one of these or-
ganisms, strain ADP9503, was confirmed by nucleotide sequencing.

Insertion mutations. The Km’ marker from pRME1 was introduced into the
EcoRV site of pZR9501, giving rise to pZR9509 (Fig. 2). This plasmid, linearized
with HindIII, was used to transform strain ADP1, and this was followed by
selection for kanamycin resistance. Sequencing of a transformant, strain ADP9502
(hcaF1::Km'), demonstrated that it had acquired

Preparation of strain ADP9510 (hcaG3::T7·TAG). A T7·TAG marker was
fused to the carboxy terminus of HcaG so that the protein could be detected
immunologically as a T7·TAG horseradish peroxidase conjugate (Novagen,
Inc., Madison, Wis.). Primers A and G were used to prepare the amplicon AG;
primers B and H were used to prepare the amplicon BH (Table 2). After restriction with EcoRI, the amplicons were ligated to form amplicon AGHB, which contained T7/H18528 TAG ligated to the carboxy terminus of HcaG (Table 2). After amplification with primers A and B, the AGHB DNA was used to transform strain ADP9500. A resulting transformant, strain ADP9510, was selected on p-hydroxybenzoate. This organism grew on chlorogenate and expressed hcaG3::T7/H18528 TAG inducibly. Sequencing demonstrated that the T7/H18528 TAG was in the predicted chromosomal location.

SDS-PAGE and immunodetection of HcaG::T7/H18528 TAG. Strain ADP9510 (hcaG3::T7/TAG) was grown to a concentration of about 5 × 10^8 cells per ml and harvested by centrifugation at 5,000 × g for 5 min. Centrifuged cells were suspended in 10 mM Na2KPO4 buffer (pH 7.0) and sonicated. Cellular debris was removed by centrifugation of the sonicated material at 5,000 × g for 15 min, and the supernatant liquid was filtered through a 2-μm-pore-size membrane. Protein concentrations were measured by the method of Bradford by using bovine serum albumin as the standard. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli (33) with a Bio-Rad mini-slab gel apparatus. Proteins from the SDS-PAGE gels were transferred to nitrocellulose by capillary action in blotting buffer (10 mM Tris, 150 mM glycine, 20% methanol; pH 8.3) for no less than 24 h. Proteins transferred to the nitrocellulose were immunostained by using horseradish peroxidase-conjugated T7/H18528 TAG antibody (Novagen) and the SuperSignal substrate (Novagen) and following the instructions of the manufacturer, which also supplied Perfect Protein Western Markers as molecular weight markers.

Separation of soluble and membrane-associated fractions. Chlorogenate-grown cultures of ADP9510 were centrifuged, resuspended in 10 mM Na2KPO4 buffer (pH 7.0), and sonicated. Whole cells were removed by centrifugation for 20 min at 5,000 × g, followed by filtration through a 2-μm-pore-size filter. The filtrate was centrifuged at 110,000 × g for 1 h at 4°C. The supernatant liquid was removed, and the pellet was suspended in a volume equal to that of the removed supernatant liquid.

Nucleotide sequence accession number. The DNA sequence of the hca genes from Acinetobacter sp. strain ADP1 have been deposited in the GenBank database under accession no. L05770. It should be noted that the designation ech has been assigned to the structural gene for enoyl-CoA hydratase lyase and the designation fcs has been assigned to the feruloyl-CoA ligase gene (1). As shown here and elsewhere (Parke, unpublished), Acinetobacter genes with these functions are closely clustered in what may prove to be a single transcript generally associated with hydroxycinnamate catabolism. The designation hca has been assigned to this transcript; hcaA is the designation for the structural gene for chlorogenate hydrolysis.
TABLE 2. Primers and amplicons

<table>
<thead>
<tr>
<th>Primer or amplicon</th>
<th>Strand</th>
<th>Position*</th>
<th>Sequence or modificationb</th>
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<tr>
<td>Primers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>+</td>
<td>400–418</td>
<td>5'-GGCGATGGCGGCCTGTGG-3'</td>
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<tr>
<td>B</td>
<td>-</td>
<td>1743–1760</td>
<td>5'-CCCCGGATTTGGGACG-3'</td>
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<td>C</td>
<td>+</td>
<td>2279–2291</td>
<td>5'-CATCACATTTGATGCGAG-3'</td>
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<td>D</td>
<td>-</td>
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<td>5'-CATAATTCCAGGCTAAAGG-3'</td>
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<tr>
<td>E</td>
<td>+</td>
<td>3013–3034</td>
<td>5'-GGTCGATTAACTGTGCGGC-3'</td>
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<tr>
<td>F</td>
<td>-</td>
<td>3630–3648</td>
<td>5'-GGTGTGAAACAGGACTGCG-3'</td>
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<tr>
<td>G</td>
<td>-</td>
<td>1069–1089</td>
<td>5'-AAAAAGAATTCATAACAGCGGATAGAAAA-3'</td>
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<td>H</td>
<td>+</td>
<td>1092–1110</td>
<td>5'-TTTTTGAATTCATAACAGCGGATAGAAAA-3'</td>
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<td>Amplicons</td>
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<tr>
<td>AB</td>
<td></td>
<td>400–1760</td>
<td></td>
</tr>
<tr>
<td>CD</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>ABCD</td>
<td></td>
<td>400–2857</td>
<td>518 bp deleted between positions 1760 and 2279 (residues 956 to 1475 in hcaG)</td>
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<tr>
<td>EF</td>
<td></td>
<td>3013–3630</td>
<td></td>
</tr>
<tr>
<td>CDEF</td>
<td></td>
<td>2279–3630</td>
<td>115 bp deleted between positions 2857 and 3013 (residues 230 to 386 in hcaF)</td>
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<tr>
<td>AG</td>
<td></td>
<td>400–1089</td>
<td>Contains poly(A) leader and EcoRI site</td>
</tr>
<tr>
<td>HB</td>
<td></td>
<td>1092–2297</td>
<td>Contains poly(T) leader and EcoRI site</td>
</tr>
<tr>
<td>AGHB</td>
<td></td>
<td>400–2297</td>
<td>Encodes T7·TAG fused to C terminus of hcaG</td>
</tr>
</tbody>
</table>

* Position relative to XhoI site in pobA.

b The chromosomal sequence is indicated by boldface type, the EcoRI sequence is italicized, and the T7·TAG sequence is underlined.

enoyl-CoA hydratase lyase (Parke, unpublished), and hcaC is the designation for the structural gene for hydroxycinnamate-CoA ligase.

RESULTS

Selection and properties of mutant strain ADP1027 (ΔhcaC) lacking a CoA ligase required for growth with hydroxycinnamic acids. Exposure of 10^9 cells from an overnight culture of strain ADP230 (ΔpcaBDK1) to 3 mM caffeate in plates containing 10 mM succinate produced about 100 colonies on each plate. After purification by streaking on succinate plates, some of the mutants grew with succinate in the presence of caffeate but not in the presence of protocatechuate. It seemed likely that these strains were blocked in the conversion of caffeate to protocatechuic acid, and this possibility was tested by replacing ΔpcaBDK1 in one of the strains with wild-type DNA and examining the growth properties of the resulting organism, strain ADP1027 (Table 1). This strain used p-hydroxybenzoate as a growth substrate and failed to grow at the expense of caffeate, p-coumarate, ferulate, and 3,4-dihydroxyphenylpropionate by converting the compounds to the corresponding thioesters (Fig. 1).

Plasmid pZR400 is known to contain pobA and a segment of DNA downstream from this gene (13). The subclone pZR9500 (Table 1) contains a portion of pobA and the previously uncharacterized downstream DNA. Transformation of strain ADP1027 with linearized pZR9500 produced recombinants that grew with caffeate. Therefore, it was evident that the gene inactivated in strain ADP1027 is part of the insert in pZR9500 and is linked to pobA. Sequencing of DNA downstream from pobA in pZR9500 revealed five open reading frames designated hcaC, hcaD, hcaE, hcaF, and hcaG (Fig. 2; Table 3). Transformation of strain ADP1027 with subclone pZR9503 (Fig. 2) showed that the mutation is within an open reading frame designated hcaC. Sequencing the corresponding portion of the mutant strain ADP1027 chromosome revealed that the selected spontaneous hcaC1 mutation is a 100-bp deletion extending from the sequence ACATT at position 702 to ACATT beginning at position 802 in the gene.

The deduced primary structure of HcaC corresponds to a protein containing 626 amino acids and exhibiting 53% sequence identity with feruloyl-CoA ligase from Pseudomonas sp. strain HR199 (47). The growth properties of strain ADP1027 indicate that the Acinetobacter CoA ligase encoded by hcaC initiates metabolism of caffeate, p-coumarate, ferulate, and 3,4-dihydroxyphenylpropionate by converting the compounds to the corresponding thioesters (Fig. 1).

Strain ADP9504 (hcaD::Kmr) lacks an acyl-CoA dehydrogenase required for conversion of 3,4-dihydroxyphenylpropionyl-CoA to caffeoyl-CoA. HcaD is a 379-amino-acid protein that is a member of a large family of flavin adenine dinucleotide-dependent acyl-CoA dehydrogenases. The knockout mutation hcaD::Kmr prevented growth with 3,4-dihydroxyphenylpropionate and allowed growth with caffeate. Since the hcaC-encoded CoA ligase is required for growth with both compounds, it is likely that HcaD oxidizes the saturated propionyl-CoA side chain of phenylpropanoyl thioesters (Fig. 1).

HcaE resembles VanP, a porin associated with vanillate metabolism in Acinetobacter. HcaE is a 416-amino-acid protein that in primary structure most closely resembles VanP of Pseudomonas aeruginosa (24). Thus, two porin genes are linked to genes associated with ferulate catabolism in Acinetobacter; one porin gene is part of the hca cluster, and the other porin gene is associated with the van genes for conver-

Table 3. Genes in the sequenced hca region

<table>
<thead>
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<th>Gene</th>
<th>Strand</th>
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<th>Deduced function</th>
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<td>hcaC</td>
<td>-</td>
<td>5830–7710</td>
<td>Hydroxycinnamate lyase</td>
</tr>
<tr>
<td>hcaD</td>
<td>-</td>
<td>4621–5760</td>
<td>Acyl-CoA dehydrogenase</td>
</tr>
<tr>
<td>hcaE</td>
<td>-</td>
<td>3295–4530</td>
<td>Porin</td>
</tr>
<tr>
<td>hcaF</td>
<td>-</td>
<td>2854–3243</td>
<td>Unknown</td>
</tr>
<tr>
<td>hcaG</td>
<td>-</td>
<td>1089–2831</td>
<td>Chlorogenic esterase</td>
</tr>
<tr>
<td>pobA</td>
<td>+</td>
<td>1–1041</td>
<td>p-Hydroxybenzoate hydratase</td>
</tr>
</tbody>
</table>
sion of vanillate, a product of ferulate, to protocatechuate (Fig. 1). The van genes are exceptional in that they occupy a genetically unstable locus far from all of the other genes required for complete catabolism of ferulate (56). In light of the structural similarity of ferulate and vanillate, it seemed likely that multiple knockout mutations would be required in order to observe the possible contributions of vanP and hcaE to ferulate metabolism, so genetic analysis of hcaE was not included as part of this study.

A knockout mutation in hcaF confers no discernible phenotype. Sequence comparisons revealed few matches for the 129-amino-acid protein predicted to be encoded by hcaF. The closest matches are with proteins with unknown functions from Brucella and Caulobacter. Knockouts caused by hcaF1::Kmr and H9004 hcaF2 had no discernible effect on catabolism of hydroxycinnamates, so the function of hcaF remains unknown.

Properties of strain ADP9501 (H9004 hcaG2) lacking an esterase required for hydrolysis of chlorogenate to quinate and caffeate. The amino acid sequence of HcaG exhibits 29 and 27% identity to the deduced primary structures of a tannase from Agrobacterium tumefaciens (60) and a feruloyl esterase from A. niger (12), respectively. A likely function for HcaG was inferred from the location of its structural gene between other hca genes required for caffeate catabolism and the qui genes associated with quinate catabolism in Acinetobacter (Fig. 2). Since hydrolysis of chlorogenate produces caffeate and quinate (Fig. 2), it seemed likely that HcaG is chlorogenate esterase. This supposition was confirmed by demonstration that the knockout mutation ΔhcaG1 eliminated the ability of Acinetobacter to grow with chlorogenate while it left the ability to grow with caffeate or quinate intact. Mutations blocking hcaF (Table 1) did not prevent growth with chlorogenate.

Regulation of chlorogenate esterase (HcaG) synthesis by intracellular catabolites and evidence that the enzyme is membrane associated. An interesting feature of the chlorogenate esterase reaction is that one of its products, quinate, is metabolized outside the inner cell membrane (9), whereas the other product, caffeate, presumably is metabolized intracellularly through the thioester intermediate. This raised the possibility that the esterase acts upon chlorogenate in the inner cell membrane and that chlorogenate does not enter cells. This consideration, coupled with the observation that hcaG is in a position where it may be cotranscribed with other hca genes, suggested that the inducer of chlorogenate esterase might be either caffeate or an intracellular catabolite formed from chlorogenate. The protein was determined by using mutant strain ADP9510 (hcaG3::T7·TAG), which produces HcaG with an epitope tag fused to its carboxyl terminus. As judged by growth properties, the in vivo activity of the mutant was unimpaired by the fusion, and the esterase protein was detected immunochemically in cell extracts. As shown in Fig. 3, HcaG3::T7·TAG was not induced by growth with quinate. Growth with chlorogenate, caffeate, ferulate, or coumarate induced the enzyme (Fig. 3). An indication that metabolism of caffeate may be necessary for induction of HcaG emerged from the observation that mutant strain ADP1027 (H9004 hcaC1), which was unable to form caffeoyl-CoA from caffeate, did not grow with chlorogenate.

The inference that chlorogenate esterase (HcaG) is membrane associated was explored by testing for the presence of HcaG3::T7·TAG in soluble and membrane fractions separated from extracts of chlorogenate-grown cultures of strain ADP9510. As shown in Fig. 3C, HcaG protein was strongly represented in the membrane fraction of cell extracts (C).

FIG. 3. Induction and cellular location of chlorogenate esterase detected as HcaG3::T7·Tag in cell extracts (A and B) and in a membrane preparation (C). The amount of protein separated by SDS-PAGE in each lane is indicated. Immunoblots show the presence of the esterase in extracts of cells grown with chlorogenate, caffeate, ferulate, and coumarate (A and B) but not in extracts of cells grown with quinate (A). The esterase was associated with the membrane fraction of cell extracts (C).
DISCUSSION

Gene organization and the natural environment. The nutritional versatility of microorganisms has been examined most frequently and most conveniently by individual presentation of potential growth substrates. Such studies have resulted in valuable conclusions about how shared sets of common nutrients help to define microbial taxa. Left unanswered are questions about where and how the nutrients are encountered by the microorganisms in their natural environments. Curiously, some clues in this regard have emerged from studies of chromosomal gene organization. A tendency towards clustering of bacterial genes for related catabolic functions has been known for some time (35, 52), and two large clusters have been identified in the chromosome of Acinetobacter sp. strain ADP1. One of these, the \textit{sal-are-ben-cat} cluster (28, 29), encodes enzymes that metabolize a range of aromatic acids, alcohols, and esters through catechol and the \(\beta\)-ketoadipate pathway.

Our knowledge concerning the other large cluster, containing the \textit{dca-pca-qui-pob} genes, was expanded to include \textit{hca} genes through this investigation. Most of the \textit{dca-pca-qui-pob-hca} genes contribute to metabolism of aromatic (2, 15) and hydroaromatic (17) compounds through protocatechuate, but the \textit{dca} genes (48) encode enzymes that act upon straight-chain dicarboxylic acids by independent metabolic pathways (Fig. 2). A shared characteristic of all the genes is that they encode pathways for catabolism of compounds that are produced by plants in response to stress. In particular, the dicarboxylic acids and hydroxyaromatic acids are components of the protective plant polymer suberin (4, 23, 38, 61). Hydroxyxycinnamate compounds serve additional protective roles, such as the contribution of ferulate in forming a “molecular spot weld” (32) in response to physical damage. This grouping of functions warranted the initial examination of cloned DNA extending downstream from \textit{pobA} for the gene inactivated by the spontaneous \textit{\textit{hcaC1}} mutation blocking conversion of a hydroxycinnamate, caffeate, to protocatechuate. The requirement for \textit{hcaC} for metabolism of ferulate, coumarate, and 3,4-dihydroxypropionate shows that the CoA ligase encoded by the gene has specificity broad enough to encompass common hydroxycinnamates. Breadth of specificity also is exhibited by plant hydroxycinnamate-CoA ligases, which are commonly designated coumarate CoA ligases (25, 34, 36, 57). Interest in the production of vanillin from ferulate has directed attention to microbial ferulate CoA ligases (1, 19, 40, 47, 58), although these enzymes act...
upon a range of hydroxycinnamates (40). Where breadth of specificity has been established and is known to contribute to biological function, it might be preferable to refer to such enzymes as hydroxycinnamate CoA ligases.

Downstream from hcaC is hcaD encoding a protein corresponding to a member of an acyl-CoA dehydrogenase family of enzymes. Inactivation of hcaD prevents growth with 3,4-dihydroxyphenylpropionate (Fig. 1). Since hcaC also is required for metabolism of 3,4-dihydroxyphenylpropionate, the overall findings are consistent with the hypothesis that HcaC has a specificity broad enough to allow formation of 3,4-dihydroxyphenyl-CoA, the substrate for the acyl-CoA dehydrogenase (Fig. 1). The genetic location of the HcaD gene suggests that hydroxycinnamates and their derivatives with saturated side chains may serve as companion nutrients for organisms related to Acinetobacter sp. strain ADP1 in their natural environments.

The chromosomal location of hcaG between the qui (quinate) and hca (hydroxycinnamate) genes for catabolism of its products (quinate and caffeate) provided an essential clue in determining the function of HcaG, which proved to be chlorogenate esterase. Had hcaG been located elsewhere on the chromosome, the effect of a knockout hcaG mutation on chlorogenate metabolism would not have been tested. Does chlorogenate accompany the hydroxycinnamates that combine to provide nutrition for Acinetobacter strain sp. strain ADP1 and related organisms in the natural environment? This possibility is heightened by evidence that hydroxycinnamate metabolism induces chlorogenate esterase. Growth with hydroxycinnamates alone would cause gratuitous synthesis of the esterase.

Left unanswered by this investigation is the range of chlorogenate substrates that are hydrolyzed by HcaG. This information, coupled with information about the distribution of chlorogenic acids in different plant sources (6, 42, 49, 59), might provide greater insight into the niche occupied by Acinetobacter sp. strain ADP1. The inference that hydroxycinnamates help to define the niche of the organism comes from the observation that the organism does not grow with tyrosine, a common amino acid that could be converted by a single enzyme (tyrosine ammonia lyase) to coumarate, a hydroxycinnamate that supports robust growth of the strain. In this sense, the organism is a nutritional specialist that is dependent upon plant products and seemingly eschews the opportunity to be a generalist which is capable of growth with a compound found in all living things.

Another question, not addressed here, is the mechanism by which the two-carbon fragment is removed from the side chain of hydroxycinnamyl thioesters during their metabolism. In another investigation, Parke (unpublished) has shown that, as reported in studies with other bacteria (1, 19, 49, 50), a hydrolase/lyase cleaves the thioesters, forming the corresponding aldehydes and acetyl-CoA in Acinetobacter sp. strain ADP1. Ferulate is catabolized through vanillate in this organism (56).

**Cellular location of chlorogenate esterase.** Chlorogenate esterase synthesis may or may not be physiologically burdensome, but its demands certainly are complex. One product of the enzyme, quinate, is metabolized outside the inner cell membrane (9), whereas the other product, caffeate, is metabolized at an intracellular location, as indicated by the requirement for CoA (Fig. 4). The simplest interpretation of this finding is that chlorogenate esterase is located within the inner cell membrane so that quinate is produced on one side and caffeate is produced on the other (Fig. 4). Alternatively, quinate and caffeate may be produced in the periplasm, followed by transport of the caffeate across the inner cell membrane. Consistent with both interpretations is the apparent location of the epitope-tagged enzyme (HcaG3::T7·TAG) in the membrane fraction of cell extracts (Fig. 3).

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**REFERENCES**


