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Hydroxycinnamate (*hca*) Catabolic Genes from *Acinetobacter* sp. Strain ADP1 Are Repressed by HcaR and Are Induced by Hydroxycinnamoyl-Coenzyme A Thioesters

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Hydroxycinnamates are plant products catabolized through the diphenol protocatechuate in the naturally transformable bacterium *Acinetobacter* sp. strain ADP1. Genes for protocatechuate catabolism are central to the *dca-pca-qui-pob-hca* chromosomal island, for which gene designations corresponding to catabolic function are *dca* (dicarboxylic acid), *pca* (protocatechuate), *qui* (quinate), *pob* (*p*-hydroxybenzoate), and *hca* (hydroxycinnamate). *Acinetobacter hcaC* had been cloned and shown to encode a hydroxycinnamate:coenzyme A (CoA) SH ligase that acts upon caffeate, *p*-coumarate, and ferulate, but genes for conversion of hydroxycinnamoyl-CoA to protocatechuate had not been characterized. In this investigation, DNA from *pobS* to an *Xba*I site 5.3 kb beyond *hcaC* was captured in the plasmid pZR8200 by a strategy that involved in vivo integration of a cloning vector near the *hca* region of the chromosome. pZR8200 enabled *Escherichia coli* to convert *p*-coumarate to protocatechuate in vivo. Sequence analysis of the newly cloned DNA identified five open reading frames designated *hcaA*, *hcaB*, *hcaK*, *hcaR*, and ORF1. An *Acinetobacter* strain with a knockout of HcaA, a homolog of hydroxycinnamoyl-CoA hydratase/lyases, was unable to grow at the expense of hydroxycinnamates, whereas a strain mutated in HcaB, homologous to aldehyde dehydrogenases, grew poorly with ferulate and caffeate but well with *p*-coumarate. A chromosomal fusion of *lacZ* to the *hcaE* gene was used to monitor expression of the *hcaABCDE* promoter. LacZ was induced over 100-fold by growth in the presence of caffeate, *p*-coumarate, or ferulate. The protein deduced to be encoded by *hcaR* shares 28% identity with the aligned *E. coli* repressor, MarR. A knockout of *hcaR* produced a constitutive phenotype, as assessed in the *hcaE*::*lacZ*-Km^r genetic background, revealing HcaR to be a repressor as well. Expression of *hcaE*::*lacZ* in strains with knockouts in *hcaA*, *hcaB*, or *hcaC* revealed unambiguously that hydroxycinnamoyl-CoA thioesters relieve repression of the *hcaABCDE* genes by HcaR.

Synthesized by plants and comprising an aromatic ring with a propenoate unit at the 1 position, hydroxycinnamates play diverse, critical roles in plant architecture and defense. They are present as structural components of cell walls, of the protective matrix suberin, and of lignin, they contribute to allelopathic interactions among plants, and they are precursors in the synthesis of flavonoids. Nutritional opportunities presented by hydroxycinnamates contribute to bacterial diversity. Particular interest has been directed to pathways of hydroxycinnamate dissimilation that generate aldehyde intermediates, such as vanillin, with biotechnological potential.

Acinetobacter sp. strain ADP1 is capable of utilizing the hydroxycinnamates ferulate, *p*-coumarate, and caffeate as sole sources of carbon and energy. The strain, derived from strain BD413, which was isolated by Elliot Juni (29), is remarkable in its capacity for high-efficiency natural transformation by DNA (36). The chromosome of strain ADP1 contains an "island of catabolic diversity" which encodes many catabolic enzymes and related proteins. Central to this genetic island are the *pca* genes, essential for catabolism of the diphenolic compound protocatechuate, along with the linked *qui* (quinate/shikimate) genes and adjacent *pob* (*p*-hydroxybenzoate) genes. Dissimilation of both quinate and *p*-hydroxybenzoate occurs via protocatechuate. At the other end of the cluster, *dca* genes for catabolism of adipate and other straight-chain, saturated dicarboxylic acids have been identified (45) (Fig. 1A).

Interest in discovering the extent of the *pca*-centered catabolic cluster led to the sequencing of plasmid pZR9500, which contains *pobA* and flanking DNA from strain ADP1. Genetic evidence revealed that downstream of *pobA* and transcribed in the opposite orientation lie genes for enzymes of hydroxycinnamate metabolism that route substrates through degradative pathways to protocatechuate (55). In addition to *hcaG*, encoding an esterase that breaks down chlorogenate to produce quinate and caffeate, the stretch beyond the *pobA* gene contains the *hcaD* gene encoding an acyl-coenzyme A (acyl-CoA) dehydrogenase homolog that enables ADP1 cells to generate hydroxycinnamoyl-CoA thioesters from corresponding compounds with a saturated propanoate side chain. Upstream of *hcaD* is *hcaC*, which encodes a hydroxycinnamate:CoASH ligase for the initial step in dissimilation of caffeate, *p*-coumarate, and ferulate. At the distal end of the insert in pZR9500 is a partial open reading frame (ORF) with similarity to aldehyde dehydrogenase genes (55) (Fig. 2).

Evidence from overflow metabolites in another *Acinetobacter* strain led to proposed pathways for the catabolism of *p*-coumarate via *p*-hydroxybenzoate and of ferulate via vanillate (13). Supporting evidence for similar metabolic routes has come from work with bacteria of other genera. Some or all of

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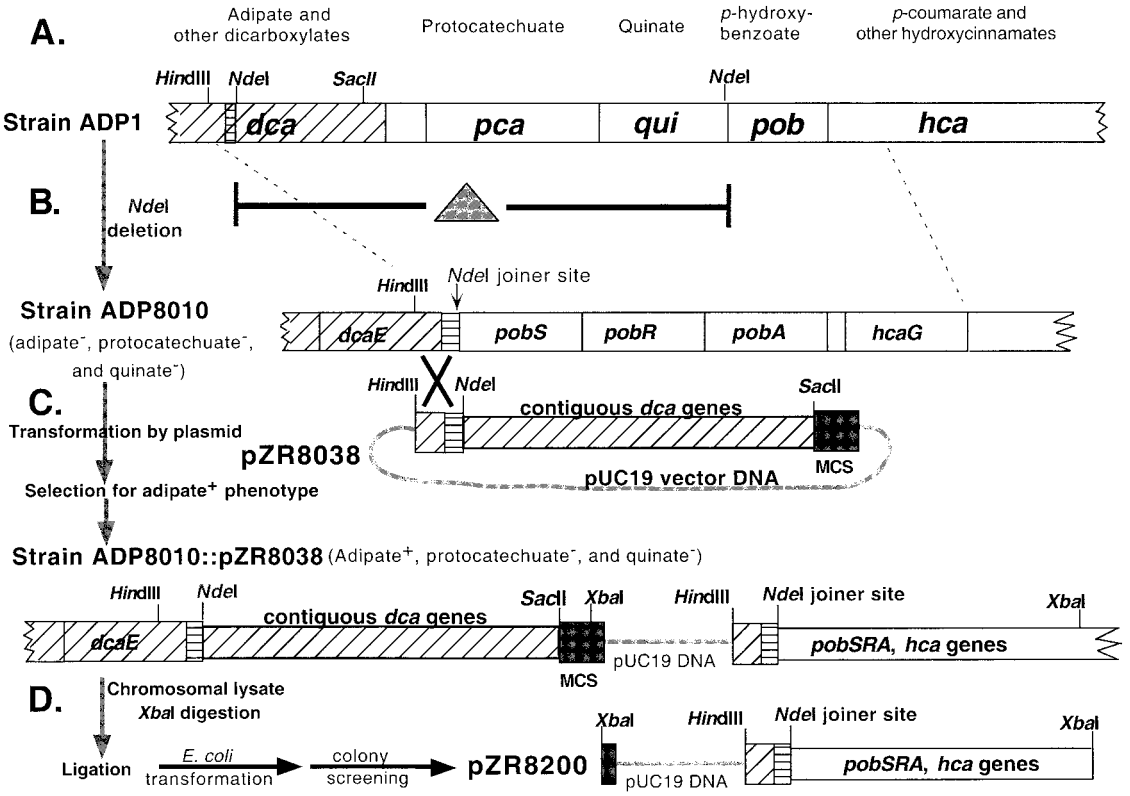

FIG. 1. Physical arrangement of the *dca-pca-qui-pob-hca* genetic cluster of *Acinetobacter* sp. strain ADP1 and the strategy used to capture the *hca* genes. (A) Organization of the genetic regions surrounding the *pca* gene cluster in the wild-type strain. (B) Introduction of an *Nde*I deletion that removed DNA from an intergenic *dca* region up to the *pobS* gene to form strain ADP8010, defective in adipate, protocatechuate, and quinate catabolism. The small segment of DNA on the left of some *Nde*I sites (▤) is part of a *dca* intergenic region. (C) Transformation of strain ADP8010 by pZR8038, followed by selection on adipate, yielding heterogenotes in which a single crossover restored the ability of cells to utilize adipate. MCS, multiple cloning site. (D) Digestion of a lysate of the heterogenote ADP8010::pZR8038 with *Xba*I followed by ligation and transformation of *E. coli*, yielding plasmid pZR8200, which was found to have a 16.5-kb insert containing all of the *pob* and *hca* genes.

the genes involved in the breakdown of ferulate to vanillate have been characterized from *Pseudomonas* sp. strain HR199 (41, 48), *Pseudomonas putida* WCS358 (59), *Pseudomonas fluorescens* AN103 (20), *Amycolatopsis* sp. strain HR167 (1), and *Sphingomonas paucimobilis* SYK6 (34).

Gasson et al. discovered that conversion of feruloyl-CoA to vanillin in *P. fluorescens* was catalyzed by an unusual, bifunctional enzyme, an enoyl-CoA hydratase/lyase (20). In spite of the potential diversity of pathways, the same type of hydratase/lyase reaction was found to mediate ferulate to vanillin conversion in *Pseudomonas* sp. strain HR199 (41), the gram-positive bacterium *Amycolatopsis* sp. strain HR167 (1), and *S. paucimobilis* (34). The pathway for hydroxycinnamate dissimilation common to these diverse microbes is shown in Fig. 3.

The *Acinetobacter* DNA insert in pZR9500 did not contain all of the genes required for conversion of hydroxycinnamates to protocatechuate. This communication describes the capture of the rest of the *hca* genes via endogenous vector integration, the newly cloned *hca* genes, the mechanism of regulatory control over them, and the structure of the inducer responsible for triggering expression of the *hca* genes.

MATERIALS AND METHODS

Strains, media, and growth of cells. Table 1 presents strains and plasmids used in this study. Cells were cultured in Luria-Bertani medium (54) or minimal medium (46). Sigma Chemical Co. and Aldrich Chemical Co. were the sources of hydroxy-*trans*-cinnamates and other growth substrates. Solidified minimal medium contained succinate at 10 mM or *p*-coumarate or ferulate at 2 mM. Comparative growth yields of *Acinetobacter* strains in liquid medium were carried out with stock solutions of substrates prepared at a concentration of 1.0 M in dimethyl sulfoxide, and hydroxycinnamates were provided in doses of 2 mM.

Luria-Bertani medium contained kanamycin and ampicillin concentrations of 15 μg ml^{-1} and 100 μg ml^{-1}, respectively, for the screening of *Acinetobacter* cells for antibiotic resistance markers. Because of natural low-level resistance of ADP1 cells to ampicillin, it was necessary to streak to isolated single colonies to screen for ampicillin resistance. Selection against cells that carried a *sacB*-Km^r cassette (27) was applied on Luria-Bertani medium containing 5% sucrose. When *Escherichia coli* cells were under selection, Luria-Bertani medium was supplemented with kanamycin or ampicillin at 25 or 100 μg ml^{-1}, respectively.

Growth was monitored by measuring turbidity as cultures were incubated at 37°C and 250 rpm. To determine growth rate constants, succinate-grown inocula in late exponential phase were diluted 20-fold into 10 ml of medium in 50 ml Erlenmeyer flasks. For comparison of lag times, succinate-grown inocula were from overnight stationary-phase cultures.

General methods of strain and plasmid construction. Molecular biology manipulations followed standard techniques (5, 51). Natural transformation of *Acinetobacter* strains was accomplished by published methods (28). Crude lysates were prepared by resuspension of pelleted cells in 500 μl of lysis buffer which contains 0.15 M NaCl, 0.015 M Na^+ citrate, and 0.05% sodium dodecyl sulfate (28), followed by incubation at 60°C for 1 h. Lysates of cells for PCRs were derived from 1 ml of a succinate overnight culture. The ability of mutant strains of ADP1 to take up DNA carried on plasmids in *E. coli* by replica plating has been described (6, 45).

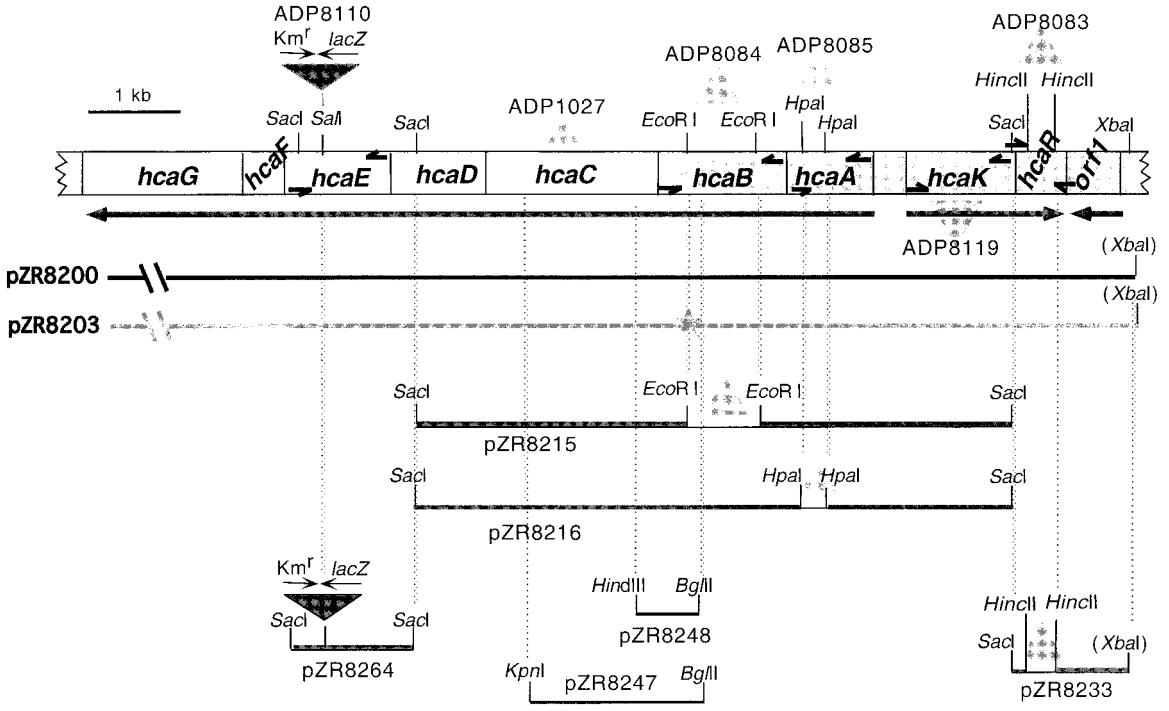

FIG. 2. Physical map of the *hca* genes showing mutations of strains and some of the subclones used in strain construction or characterization. Shading indicates the newly captured *hca* DNA present in pZR8200. *Acinetobacter hca* DNA on plasmid pZR9500, characterized in a previous study (55), ended near the 3′ terminus of *hcaB*, where the shading begins. The star on pZR8203 marks the location of a frameshift mutation. Arrows denote direction of transcription but are not necessarily transcriptional units, triangles mark the sites of deletion mutations, and half-arrows mark the sites of primers used to verify mutations.

Enzyme assays. LacZ assays were conducted according to the method of Miller (37). *Acinetobacter* cells containing an *hcaE*::*lacZ*-Km^r mutation were grown overnight in minimal medium containing 10 mM succinate, 100 μl was inoculated into 5 ml of fresh succinate medium with or without an aromatic compound, and cells were grown at 250 rpm and 37°C for 4.5 h. Cultures were harvested as previously described (44). In the special case of ADP8110 cells grown at the expense of hydroxycinnamates in the absence of succinate, cells were grown under similar conditions, but the inocula, cells in late exponential phase of growth on succinate, were diluted only 10-fold. The latter cells were grown at the expense of 4 mM caffeate, *p*-coumarate, or ferulate, each provided in separate doses of 2 mM.

Cloning the DNA upstream of *hcaC*. Figure 1 outlines the strategy used to clone DNA upstream of the *hcaC* gene. Plasmid pZR8000 (45) contains a large insert spanning *dca*, *pca*, *qui*, and *pob* genes. A 25.8-kb *Nde*I deletion in pZR8000 removed part of an intergenic region between two divergently transcribed *dca* operons, as well as all intervening DNA up to the *pobS* gene (Fig. 1B). Introduction of the *Nde*I deletion into strain ADP1 created strain ADP8010, which was unable to utilize protocatechuate, quinate, or the dicarboxylate adipate.

Plasmid pZR8038 contains an insert of *Acinetobacter dca* DNA, which includes 1 kb of DNA downstream from the *dca* intergenic *Nde*I site; the 1-kb region is the only *Acinetobacter* DNA segment in the insert homologous to the chromosome of Δ*Nde*I strain ADP8010. Cells of ADP8010 were transformed with pZR8038, and 200 μl of the transformant culture was inoculated into 5 ml of liquid minimal medium supplemented with 5 mM adipate. The rationale for the adipate selection is that, theoretically, growth of ADP8010 on adipate can be accomplished only by integration of pZR8038 into the chromosome by means of a single crossover at the 1-kb homologous region of DNA, as shown in Fig. 1C. After overnight incubation, the cells were centrifuged and resuspended in lysis buffer, and a lysate was prepared. Digestion of the chromosomal lysate with *Xba*I was followed by precipitation, threefold dilution, heat inactivation, ligation, concentration, and transformation of *E. coli* DH5α cells chemically prepared to be highly competent (24).

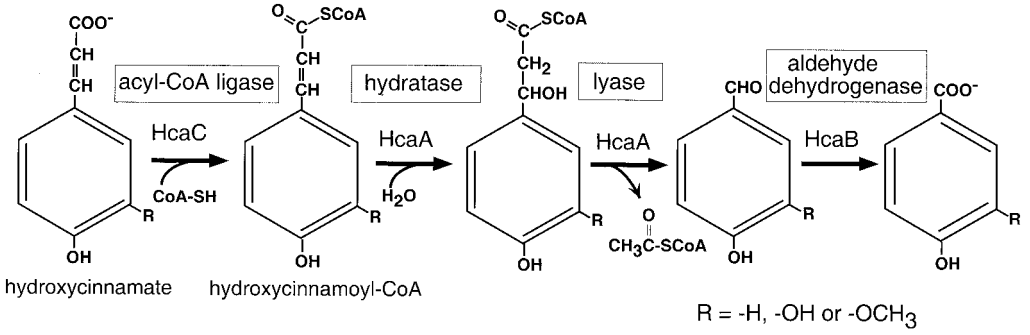

FIG. 3. Pathway predicted to transform the hydroxycinnamates *p*-coumarate, caffeate, and ferulate to the respective intermediates *p*-hydroxybenzoate, protocatechuate, and vanillate in *Acinetobacter* sp. strain ADP1. Homologs of HcaA, shown as two reaction products, have been shown to be bifunctional enzymes that participate in the metabolism of ferulate by other organisms, as noted in the text.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Genotype	Reference or source
E. coli DH5α	F$^-$ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17* (r_K^- m_K^-) *supE44* λ$^-$ *thi-1 gyrA96 relA1*		Invitrogen
Acinetobacter sp. strains			
ADP1	Wild-type strain (also known as BD413)		29
ADP992	90-bp deletion in *pobA*; block in *p*-hydroxybenzoate hydroxylase	Δ*pobA*992	D. A. D'Argenio, unpublished data
ADP1027	100-bp deletion in *hcaC*; block in hydroxycinnamate: CoASH ligase	Δ*hcaC*1	55
ADP8010	25.8-kb *Nde*I deletion from pZR8033 with endpoints in a *dca* intergenic region (between *dcaECHF* and *dcaAKIJPQ*) and the *pobS* gene; genes deleted include those required for dicarboxylate, protocatechuate, and quinate catabolism	Δ(*dca-quiA*)1	This study
ADP8078	Km^r; Δ (*hcaR orf1*)::*sacB*-Km^r from pZR8231 introduced into the ADP1 chromosome	Δ(*hcaR-orf1*)::*sacB*-Km^r	This study
ADP8079	Km^r; Δ(*hcaKAB*)::*sacB*-Km^r from pZR8227 introduced into the ADP1 chromosome	Δ(*hcaKAB)1*::*sacB*-Km^r	This study
ADP8081	Km^r; Δ*hcaB1*::*lacZ*-Km^r from pZR8238 in the chromosome of ADP1	Δ*hcaB1*::*lacZ*-Km^r	This study
ADP8083	217-bp *Hinc*II deletion in *hcaR* from pZR8233 in the ADP8078 chromosome	Δ*hcaR1*	This study
ADP8084	600-bp *Eco*RI deletion in *hcaB* from pZR8215 in the chromosome of ADP8079	Δ*hcaB3*	This study
ADP8085	163-bp *Hpa*I deletion in *hcaA* from pZR8216 in the chromosome of ADP8079	Δ*hcaA1*	This study
ADP8088	Km^r; Δ(*hcaR-orf1*)::*sacB*-Km^r from pZR8231 in the chromosome of ADP8083	Δ*hcaR1* Δ*hcaB1*::*lacZ*-Km^r	This study
ADP8101	Km^r; Δ(*hcaRorf1*)::*sacB*-Km^r from pZR8246 in the chromosome of ADP1027	Δ*hcaC1* Δ(*hcaR-orf1*)::*sacB*-Km^r	This study
ADP8102	Km^r; *hcaK*::*sacB*-Km^r from pZR8246 in ADPI background	*hcaK1*::*sac*-Km^r	This study
ADP8110	Km^r; *hcaE*::*lacZ*-Km^r from pZR8264 in the chromosome of ADP1	*hcaE1*::*lacZ*-Km^r	This study
ADP8113	Km^r; *hcaE*::*lacZ*-Km^r from pZR8264 in the chromosome of ADP1027	Δ*hcaC1 hcaE1*::*lacZ*-Km^r	This study
ADP8114	Km^r; *hcaE*::*lacZ*-Km^r from pZR8264 in the chromosome of ADP8085	Δ*hcaA1 hcaE1*::*lacZ*-Km^r	This study
ADP8116	Km^r; *hcaE*::*lacZ*-Km^r from pZR8264 in the chromosome of ADP8084	Δ*hcaB3 hcaE1*::*lacZ*-Km^r	This study
ADP8117	Km^r; *hcaE*::*lacZ*-Km^r from pZR8264 in the chromosome of ADP8083	Δ*hcaR1 hcaE1*::*lacZ*-Km^r	This study
ADP8119	Km^r; Δ*hcaK* from pZR8271 in the chromosome of ADP8102	Δ*hcaK*3	This study
ADP8121	Δ*hcaR1* from pZR8233 in the chromosome of ADP8101	Δ*hcaC1* Δ*hcaR1*	This study
ADP8135	Δ(*hcaRKABCDEFG*) from pZR8207 in the chromosome of ADP8102; hydroxycinnamate negative	Δ(*hcaRKABCDEFG*)1	This study
ADP8136	Km^r; *hcaE*::*lacZ*-Km^r from pZR8264 in the chromosome of ADP8121	Δ*hcaC1* Δ*hcaR1* *hcaE1*::*lacZ*-Km^r	This study
Plasmids			
pBKS	Ap^r; narrow host range cloning vector		Stratagene
pBKSΔ1	Ap; pBKS which is Δ*Sma*I-*Hinc*II		This study
pKOK6	Ap^r Km^r Tc^r; contains 4.6-kb promoterless *lacZ*-Km^r cassette for constructing operon fusions		31
pRMJ1	Ap^r Km^r; contains *sacB*-Km^r cassette for genetic replacement by positive selection		27
pRK415	Tc^r; broad-host-range vector		30
pUC4K	Ap^r Km^r; contains antibiotic resistance cassette		Amersham Pharmacia Biotech
pUC18 and pUC19	Ap^r; narrow-host-range cloning vectors		61
pZR8000	Chl^r; 34.8-kb *Acinetobacter* sp. strain ADP1 *Sac*I insert in pBBR1MCS containing *dca*, *pca*, *qui*, and *pob* genes		45
pZR8033	Chl^r; 25.8-kb *Nde*I deletion in pZR8000		This study
pZR8038	Ap^r; 8.9-kb *Hind*III-*Sac*II insert of *dca* genes from pZR8000 in pUC19		45
pZR8200	Ap^r; 16.5-kb *Hind*III-X*ba*I insert of DNA from strain ADP8010::pZR8038, which encompasses 1-kb of *dca* DNA, the *pob* and *hca* genes; in pUC19		This study
pZR8203	Ap^r; pZR8203 with a mutation introduced at the unique *Xcm*I site in *hcaB*		This study
pZR8207	Δ*hcaRKABCDEFG* of pZR8200 created by exonuclease activity of Klenow fragment		This study
pZR8210	Ap^r; 6.5-kb *Sac*I insert with *hca* genes from pZR8200 in pBKSΔ1		This study
pZR8215	Ap^r; 600-bp *Eco*RI deletion of *hcaB* in pZR8210		This study
pZR8216	Ap^r; 163-bp *Hpa*I deletion of *hcaA* in pZR8210		This study
pZR8219	Ap^r; 1.66-kb *Sac*I subclone of pZR8200 in pBKSΔ1		This study

Continued on following page

TABLE 1—*Continued*

Strain or plasmid	Relevant characteristics	Genotype	Reference or source
pZR8220	Ap^r; 2.4-kb *Pst*I deletion from *hcaB* to *hcaK* in pZR8210		This study
pZR8224	Ap^r; 1.6-kb *Sac*I subclone of pZR8200 containing *hca*R and ORF1 in pBKSΔ1		This study
pZR8227	Ap^r; Km^r; *sacB*-Km^r cassette of pRMJ1 inserted at the site of the *Pst*I deletion in pZR8220		This study
pZR8231	Ap^r; *sacB*-Km^r cassette of pRMJ1 inserted at the site of a 0.63-kb *Bgl*II deletion in pZR8224		This study
pZR8233	Ap^r; 217-bp *Hinc*II deletion within *hcaR* in pZR8224		This study
pZR8238	Tc^{r}Km^r; *lacZ*-Km^r cassette from pKOK6 in the Δ*Bgl*II site of *hcaB* in pZR8223; *lacZ* expression is in the same direction as that of *hcaB*		This study
pZR8239	Tc^r; 1.2-kb *Hin*dIII-*Sac*I insert of *hcaK′* in pRK415		This study
pZR8240	Tc^r; 3.3-kb *Sac*I-*Bgl*II insert of *hcaC* plus the 3′ part of *hcaB*		This study
pZR8246	Tc^r Km^r; pRMJ1 *sacB*-Km^r cassette in *Pst*I site of *hca*K in pZR8239		This study
pZR8247	Ap^r; 2.1-kb *Kpn*I-*Bgl*II subclone of pZR8240 in pUC18		This study
pZR8248	Ap^r; 0.95-kb *Hin*dIII-*Bgl*II subclone of pZR8240 which includes 268 bp of *hcaC* and part of *hcaB* in pUC18		This study
pZR8264	Ap^r Km^r; *lacZ*-Km^r cassette from pKOK6 in the *Sal*I site of *hcaE* in pZR8219; *lacZ* expression is in the same direction as that of *hcaE*		This study
pZR8265	Ap^r; 1.2-kb *Hin*dIII-*Sac*I insert of pZR8239 in pUC18		This study
pZR8271	Ap^r; 775-bp *Bsr*GI deletion within *hcaK* in pZR8265		This study

Colonies that appeared on the vector-specific antibiotic plates were replica plated onto additional plates for further testing. They were screened for the presence of the *pobA* gene by transformation of the *Acinetobacter pobA* mutant strain ADP992. Their ability to convert *p*-coumarate to protocatechuate was screened by replica plating colonies onto Luria-Bertani antibiotic medium containing 1 mM *p*-coumarate as well as ferric chloride and *p*-toluidine, which is chromogenic for diphenolics (43).

Construction of *hca* mutant strains of ADP1. Mutations in *hca* genes were designed as deletions in order to minimize the possibility of downstream transcription termination effects which were noted with the pUC4K Km^r marker in *dca* genes (45), to allow selection for a *lacZ*-Km^r cassette, and to facilitate the eventual construction of multiply mutated strains. Strains carrying a *sacB* cassette fail to grow in the presence of 5% sucrose, and deletion strains were constructed by replacement of the *sacB*-Km^r cassette of pRMJ1 (27) in the targeted site of recipient strains as summarized in Table 1. It was necessary to do the *sacB* selection at room temperature (27), and colonies that grew in the presence of 5% sucrose were screened for the absence of the Km^r marker present in the cassette as well as absence of the vector antibiotic resistance marker.

Table 1 details the construction of strains used in this investigation. Strain ADP8083 (Δ*hcaR1*) was created by transformation of the competent *sacB*-Km^r strain ADP8078 with plasmid pZR8233 (Table 1; Fig. 2). Strains ADP8084 (Δ*hcaB3*) and ADP8085 (Δ*hcaA1*) were created by transformation of competent ADP8079 cells with plasmids pZR8215 and pZR8216, respectively (Table 1; Fig. 2). Strains ADP8110, ADP8113, ADP8114, ADP8116, and ADP8117 contain *hcaE*::*lacZ*-Km^r in different genetic backgrounds, as described in Table 1. Insertion and deletion mutations in the *Acinetobacter* chromosome were confirmed by PCR (Fig. 2).

PCRs. PCR products were produced by using dilute chromosomal lysates as templates. 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 (as possible, noted in Fig. 2) were as follows: MGZR80T38 and MGZR80F7 for Δ*Nde*I in ADP8010; DPZR82R7 and DPZR82F2 for analysis of Δ*hcaR1* in ADP8083 and ADP8136; PPABR1 and DPZR82F8 for Δ*hcaB3* in ADP8084; DPZR82R3 and DPZR82F6 for Δ*hcaA1* in ADP8085; DPZR82R5 and DPZR82F3 for Δ*hcaK3* in ADP8119; and VD902R3 and MS902F1 for *hcaE*::*lacZ*-Km^r in ADP8110, ADP8113, ADP8114, ADP8116, ADP8117, and ADP8136. Oligonucleotide sequences of these primers are as follows: MGZR80T38, 5′-ATAGTAGATTGCTATAGCG AAATATAGAGA-3′; MGZR80F7, 5′-CCTGCTTGTAATCCAGTGAGATG A-3′; DPZR82R7, 5′TCAGAAAGCCCAAACACCCTGTA-3′; DPZR82F2, 5′-TTCTTCACCATTAATTCAAGGTTATTAC-3′; PPABR1, 5′-TGCTTCAT CGTGTACGGTTGCACC-3′; DPZR82F8, 5′-CAAAATACCGAAAAGTTTA TCGAAATC-3′; DPZR82R3, 5′-TCTTCATTCTGATCCCATGTCAGCT-3′; DPZR82F6, 5′-GGGTCACACTTAATCGTCCTCACAA-3′; DPZR82R5, 5′-G AATGCGTGCCGAATTTATGTTAGA-3′; DPZR82F3, 5′-ACAGGGTGTTT GGGCTTTCTGAC-3′; VD902R3, 5′-CGATGAGCAGTCCTGTTTCACC-3′; and MS902F1, 5′-GAAGGTGTTGAAAAAGGACGTTCG-3′.

DNA sequencing. The region of pZR8200 that contained newly cloned *Acinetobacter* DNA (Fig. 2) was sequenced by primer walking at the Yale Keck Biotechnology Resource Lab. Standard ABI PRISM terminator cycle sequencing with AmpliTaq DNA polymerase was used.

Nucleotide sequence accession number. Accession no. L05770 in the GenBank database contains the DNA sequence for the *hca* genes from *Acinetobacter* sp. strain ADP1 up to an *Xba*I site.

RESULTS

Isolation of clone pZR8200. Two factors influenced the vector integration cloning strategy for *Acinetobacter*. The fact that commonly used plasmids with the ColE1 origin of replication are able to replicate in ADP1 (22) is a disadvantage when the goal is to create heterogenotes. A second consideration is that most of the *Acinetobacter* strains used in our investigations are not cleanly sensitive to ampicillin and certain other drugs used for plasmid selections. By using a recipient *Acinetobacter* strain with a designed deletion and correcting that deletion through heterogenote formation alone (Fig. 1), it was possible to apply a nutritional selection for formation and maintenance of the heterogenote. A lysate of the heterogenote-containing strain provided a source of material for cloning the full suite of *hca* genes in *E. coli*.

As summarized in Fig. 1 and described in detail in Materials and Methods, strain ADP8010, which is missing DNA from an intergenic *dca* region up to the *pobS* gene by virtue of a large *Nde*I deletion, was transformed with plasmid pZR8038. This plasmid contains the *dca* genes deleted from strain ADP8010 as well as the adjacent 1-kb region of *dca* DNA, which provided a region of homology between pZR8038 and ADP8010 for crossover to occur. Only cells that had undergone heterogenote formation could grow at the expense of adipate (Fig. 1C). Following transformation of ADP8010 with pZR8038, selection for heterogenotes on adipate, and preparation of a lysate of them, the lysate was digested with *Xba*I. The *Xba*I fragments were circularized with ligase and used to transform

E. coli. Selection for Ap^r yielded colonies that were screened for the presence of the *pobA* gene, which was not present in plasmid pZR8038 (Fig. 1C). Of the screened *E. coli* colonies, 6% were $pobA^+$; the others were presumed to have been transformed with pZR8038, which was carried by the ADP8010 transformants. All of the $pobA^+$ colonies also converted *p*-coumarate to protocatechuate as judged by accumulation of a characteristic deep purple color around colonies in a *p*-toluidine test for diphenolics (43). Plasmid pZR8200 (Fig. 1 and 2), which included 4.8 kb of uncharacterized DNA, was isolated from one of the positive colonies.

Sequence analysis of the new *Acinetobacter* DNA captured in pZR8200. The newly captured DNA of pZR8200 (Fig. 2) was deduced to contain five new ORFs that were analyzed using the BLAST program (4) against the National Center for Biotechnology Information database. Upstream of *hcaC* by 66 bp is a 1.45-kb ORF, designated *hcaB*, transcribed in the same direction, which encodes a protein with 59% identity to the aligned vanillin dehydrogenase from *Pseudomonas* sp. strain HR199 (accession no. CAA72286) (48). The sequencing of plasmid pZR9500 had uncovered 446 bp at the 3′ end of this ORF (55). The protein encoded by *hcaB* is presumed to be a dehydrogenase that acts upon aldehyde intermediates in hydroxycinnamate catabolism.

Upstream of *hcaB* by 36 bp is a 0.83-kb ORF termed *hcaA* which is positioned at the beginning of the *hca* structural gene transcript (Fig. 2). A homolog of hydroxycinnamoyl-CoA hydratase/lyases, HcaA is identical at 79% of the residues when aligned with the homolog from *P. fluorescens* (accession no. CAA73502) (20).

An intergenic distance of 256 bp separates *hcaA* from its upstream, divergently transcribed neighbor, an ORF deduced to be 1.23 kb and termed *hcaK*. HcaK is homologous to the aromatic compound transporters PcaK (accession no. Q51955 and AAC37151) (11, 23, 32), BenK (accession no. 030513) (9), and VanK (accession no. AAC27108) (52). These transporters are members of the aromatic acid transporter subgroup within the major facilitator superfamily, which displays considerable divergence among its members (42). PcaK and VanK from ADP1, both of which mediate transport of protocatechuate (11), share 31% identity along their polypeptides, while HcaK has 29 and 24% identity, respectively, with them. Typical for members of the family, HcaK is predicted to possess 12 transmembrane helices.

Translation of an ORF termed *hcaR*, which lies 63 bp downstream of *hcaK*, generates a protein with similarity to the *E. coli* repressor MarR (accession no. P27245) (8). HcaR is 159 amino acid residues long, compared to 144 residues for MarR, and these proteins share 28% identical residues along the aligned portions. Although a common Shine-Dalgarno sequence is absent upstream of the predicted ATG start codon for *hcaR*, the sequence AAG 7 bp upstream of the ATG is presumed to comprise a ribosomal binding domain (60).

Transcribed towards *hcaR* is ORF1, whose deduced translation product is a 205-residue protein. The putative translational start codon for ORF1 is TTG (12), 7 bp downstream from a GGA sequence predicted to be part of the Shine-Dalgarno sequence. ORF1 possesses 45% sequence similarity and 26% identity with the aligned primary sequence of the *E. coli* repressor TetR (accession no. S07359) (58). The *Xba*I site at the end of the insert in pZR8200 is situated 272 bp from the 5′ end of ORF1.

TABLE 2. Growth of wild-type and mutant strains with hydroxycinnamates

Strain	Mutation	Growth rate constant (h^{-1}) for growth with[a]:			
		Succinate	Caffeate	*p*-Coumarate	Ferulate
ADP1	None	1.55	0.44	0.58	0.32
ADP8083	Δ*hcaR1*	1.55	0.54	0.64	0.19
ADP8084	Δ*hcaB3*	ND	0.30	0.58	<0.09
ADP8085	Δ*hcaA1*	ND	<0.09	<0.03	<0.09

[a] ND, not determined.

Phenotypes of *hcaA*, *hcaB*, *hcaK*, and *hcaR* deletion strains. Since pZR8200 conferred upon *E. coli* the ability to convert *p*-coumarate to protocatechuate, it can be deduced that the hydratase/lyase (HcaA) and aldehyde dehydrogenase (HcaB) homologs act upon *p*-coumaroyl-CoA and *p*-hydroxybenzaldehyde, respectively.

Further analysis of the roles of HcaA and HcaB in hydroxycinnamate dissimilation was carried out following creation of *Acinetobacter* strains with deletions of each gene. Deletions (Fig. 2; Table 1), designed to totally eliminate activity of the gene products, were predicted to produce truncated versions of the wild-type proteins: 27% of HcaA, 59% of HcaB, 39% of HcaK, and 17% of HcaR. Strain ADP8085 (Δ*hcaA1*) is unable to grow at the expense of *p*-coumarate, ferulate, or caffeate (Table 2; Fig. 4).

Strain ADP8084 (Δ*hcaB3*) grows poorly at the expense of ferulate and has a leaky phenotype on caffeate and an almost wild-type phenotype on *p*-coumarate (Table 2; Fig. 4). Impairment of strain ADP8084 on *p*-coumarate, relative to that of ADP1, is manifested in a slight growth lag out of stationary phase and reduced final cell density. A likely source of the

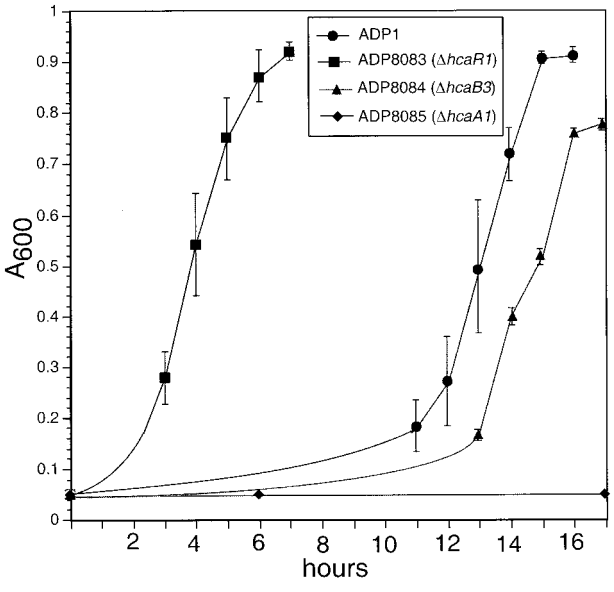

FIG. 4. Growth curves of strains utilizing *p*-coumarate as the sole carbon source. The growth curves of cultures used as inocula, grown in parallel on succinate to stationary phase, were superimposable. Error bars show the standard deviations for three independent data sets.

leaky phenotypes is the activity of aldehyde dehydrogenase(s) associated with other pathways, such as the one that acts on benzaldehyde (26).

Additional genetic tests were carried out to confirm that HcaB indeed acts on a metabolite of *p*-coumarate. To rule out the possibility that the *E. coli* DH5α(pZR8200) bioconversion of *p*-coumarate to protocatechuate depends upon an *E. coli* aldehyde dehydrogenase that can fill in for HcaB, an *hcaB* mutation in pZR8200, making plasmid pZR8203, was used. As assessed by the *p*-toluidine test (43), *E. coli* carrying pZR8203 produces protocatechuate from *p*-hydroxybenzoate but fails to produce it from *p*-coumarate. Further proof that the only relevant pZR8203 mutation blocking the *p*-coumarate to protocatechuate pathway is the mutation in *hcaB* came from the ability of pZR8203 to transform strain ADP8135 (Δ*hcaRKABCDEFG*) to a *p*-coumarate-positive growth phenotype.

Although sequence similarities to transporters of aromatic compounds suggest that HcaK is involved in transport of hydroxycinnamates across the inner cell membrane, Δ*hcaK3* mutant strain ADP8119 exhibited an unaltered phenotype when grown at the expense of 2 mM *p*-coumarate, ferulate or caffeate. The role of *hcaK* in transport of hydroxycinnamates at lower concentrations is under further investigation. A strain carrying a deletion in ORF1 exhibited a growth phenotype similar to that of ADP1.

Strain ADP8083 (Δ*hcaR1*) grew well at the expense of *p*-coumarate, caffeate, and ferulate (Table 2). In a further examination of growth kinetics, succinate-grown, stationary-phase cultures of strains ADP1 and ADP8083 were inoculated into medium containing *p*-coumarate as the sole carbon source. Another aliquot of the cells was inoculated into succinate to serve as a control for the possibility that the initial viable cell numbers were different for the two strains. Whereas the strains thus inoculated had superimposable kinetics on succinate (data not shown), the *hcaR* knockout mutant strain ADP8083 had a greatly reduced lag time compared to ADP1 when inoculated into *p*-coumarate (Fig. 4), consistent with the phenotype of a mutant strain that is constitutive for enzymes of *p*-coumarate catabolism.

Analysis of the role of *hcaR*. Further analysis of the *hcaR* mutant phenotype was conducted in cells that contained a promoterless *lacZ*-Km^r cassette in the chromosomal *hcaE* gene (Fig. 2). The product of this gene is an apparent porin precursor that is not required for growth with hydroxycinnamates at 2 mM, as used in this investigation. Figure 5 presents data on the expression of *lacZ* in cells grown for 4 h at the expense of succinate alone or with succinate plus a hydroxycinnamate. Strain ADP8110 (*hcaE*::*lacZ*-Km^r in a wild-type background) expressed at least a 100-fold increase in LacZ in response to the addition of *p*-coumarate, ferulate, or caffeate. In the presence or absence of hydroxycinnamate, regulatory mutant strain ADP8117 (Δ*hcaR hcaE*::*lacZ*-Km^r) expressed LacZ at a level that was 300-fold higher than that in succinate-grown ADP8110 cultures (Fig. 5). It should be noted that ADP8110 cells grown at the expense of hydroxycinnamates in the absence of succinate had somewhat higher LacZ levels: 930 Miller units when grown at the expense of *p*-coumarate alone, 900 Miller units when grown at the expense of caffeate alone, and 830 Miller units when grown at the expense of ferulate alone.

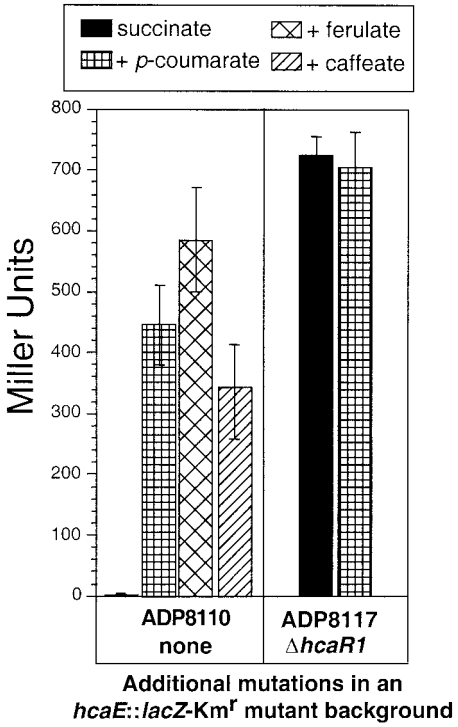

FIG. 5. Role of HcaR in expression off the *hca* promoter. Promoter activity was measured by β-galactosidase activity (Miller units) by using strains with the chromosomal insertion *hcaE*::*lacZ*. Cultures were grown on succinate with or without the addition of 1 mM caffeate, *p*-coumarate, or ferulate. Not shown are the LacZ levels for ADP8110 cells grown at the expense of hydroxycinnamates in the absence of succinate: 928 Miller units with *p*-coumarate alone, 899 Miller units with caffeate alone, and 825 Miller units with ferulate alone. Data are averages based on three independent trials; error bars indicate standard deviations.

Introduction of Δ*hcaR* into the chromosome entailed selection for loss of a *sacB*-Km^r cassette, and this was accomplished by transformation with pZR8233 (Fig. 2), which contained the *hcaR1* deletion. Thus, there was no selection or screening related to the resultant constitutive phenotype. Reintroduction of the wild-type *hcaR* gene by transformation of ADP8117 cells with pZR8224, the parental plasmid of Δ*hcaR* plasmid pZR8233, eliminated the constitutive phenotype and restored inducibility; transformation with pZR8233 yielded no such transformants.

As noted in the sequence analysis section above, ORF1 is homologous to the *E. coli* repressor TetR. If this gene encodes a repressor of *hca* genes, its knockout would be expected to produce a constitutive phenotype; if it encodes an activator of *hca* genes, its elimination should cause a hydroxycinnamate-negative phenotype. A strain bearing *hcaE*::*lacZ*-Km^r and a deletion in ORF1 displayed the wild-type growth phenotype on hydroxycinnamates, and it had a wild-type phenotype on succinate plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) plus caffeate, *p*-coumarate, or ferulate (D. Parke, unpublished data), leading to the conclusion that ORF1 is not an *hca* gene.

Identification of the inducer responsible for derepression of the *hca* genes. In order to determine the nature of the inducer

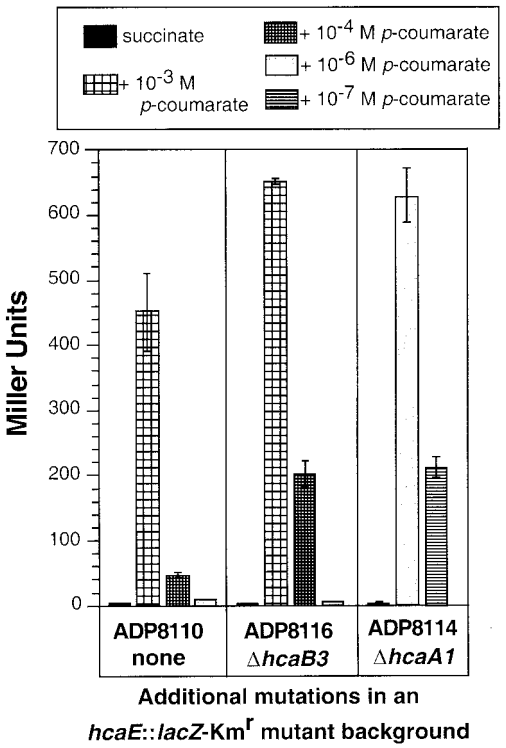

FIG. 6. Identification of *p*-coumaroyl-CoA as a true inducer of the *hca* genes. Cultures were grown on succinate with the addition of compounds as noted. Not shown because they would compress the uninduced bars are results for strain ADP8114 grown in the presence of 10^{-4} M *p*-coumarate (1,160 Miller units of LacZ activity). Promoter activity was measured, and the averages of three independent trials are presented with their standard deviations.

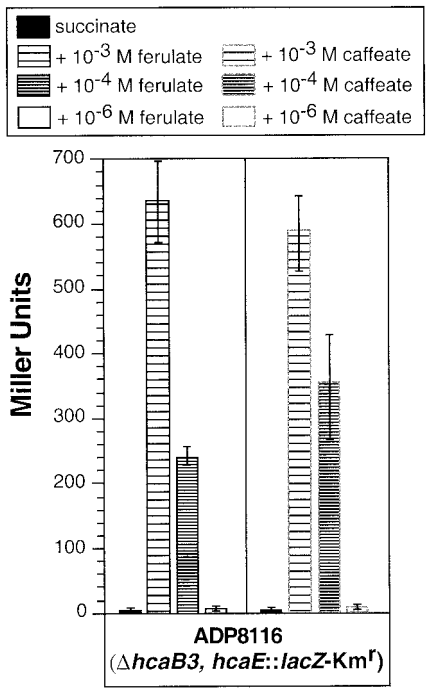

FIG. 7. Absence of induction at low concentrations of caffeate or ferulate in strain ADP8116, with a block in HcaB, the aldehyde dehydrogenase. Cultures were grown on succinate with the addition of compounds as noted. ADP8116 cells grown in the presence of 1 mM vanillin, a catabolite of ferulate, had LacZ levels close to those grown on succinate alone (data not shown). Promoter activity was measured, and the averages of three independent trials are shown with their standard deviations.

that governs the *hca* genes, the *hcaE*::*lacZ*-Km^r construction was introduced into strains in which different steps of hydroxycinnamate dissimilation were blocked.

Strain ADP8114 (Δ*hcaA1 hcaE*::*lacZ*-Km^r), in which enoyl-CoA hydratase/lyase was blocked, expressed high levels of LacZ activity in the presence of as little as 10^{-6} M *p*-coumarate, whereas LacZ activity in strain ADP8110, which possessed a wild-type background, fell dramatically when the concentration of *p*-coumarate was reduced from 10^{-3} to 10^{-4} or 10^{-6} M (Fig. 6). The results for strain ADP8116 (Δ*hcaB3 hcaE*::*lacZ*-Km^r) were similar to those for ADP8110 (Fig. 6). Because ADP8116 exhibits an impaired growth phenotype only when grown at the expense of ferulate or, to a lesser extent, caffeate, the LacZ data for ADP8116 cells induced in the presence of these two hydroxycinnamates are presented in Fig. 7. Unlike the ADP8114 results, no induction occurred at the low concentration. Moreover, the aldehyde catabolite vanillin, tested as an inducer of *hcaE*::*lacZ*, did not elicit LacZ expression in strain ADP8116 (Δ*hcaB hcaE*::*lacZ*-Km^r) (data not shown).

Evidence pointing to hydroxycinnamoyl-CoA thioesters as inducers of the *hca* genes was supported by results of experiments with cells blocked in the activation of hydroxycinnamates. Strain ADP8113 (Δ*hcaC1 hcaE*::*lacZ*-Km^r) possessed no LacZ activity in response to the presence of *p*-coumarate (Fig. 8). To confirm that the lack of expression was due to the ligase mutation and not to instability related to the *hcaE*::*lacZ*-Km^r construction or its expression, strain ADP8113 was subjected to separate transformations by pZR8247, which carries most of the ligase gene, and pZR8248, which carries a truncated version (Fig. 2). Struck to single colonies on a plate containing X-Gal, the ADP8113 population transformed with pZR8247 gave rise to blue, growing colonies on medium with 2 mM *p*-coumarate, and LacZ$^+$ blue colonies in a field of white ones were observed on medium containing succinate plus *p*-coumarate. The ADP8113 population transformed with pZR8248, the negative control, failed to grow at the expense of *p*-coumarate alone or to show blue color on either medium.

The low LacZ activity with strain ADP8113 could also be interpreted as being caused by a polar effect of the frameshift created by Δ*hcaC1* on expression of downstream genes in an operon (2). Strain ADP8136 (Δ*hcaC1* Δ*hcaR1 hcaE*::*lacZ*-Km^r) differs from ADP8113 in having an *hcaR* knockout, thus making expression of LacZ constitutive. As shown in Fig. 8, constitutive LacZ expression of ADP8136 cells is greatly reduced from that found in ADP8117, indicating that polarity caused by the Δ*hcaC1* mutation indeed compromises expression of *hcaE*::*lacZ*. If *p*-coumarate itself were an actual inducer, cells of ADP8113 should exhibit an induced level similar to the constitutive level in ADP8136. The total absence of LacZ activity in ADP8113 indicates that the unactivated compound does not have an inductive role.

Further support for this conclusion was obtained with strains

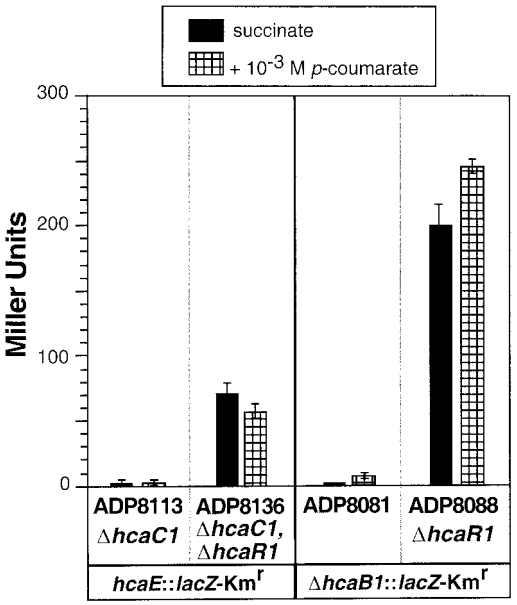

FIG. 8. Failure of unactivated *p*-coumarate to elicit expression of *hcaE*::*lacZ* or *hcaB*::*lacZ* in strains impaired in hydroxycinnamate:CoASH ligase. Succinate-grown cells were amended with *p*-coumarate as noted. LacZ levels for the two constitutive strains with the Δ*hcaR1* mutation provide the respective values to be expected for induced cells analogous to what was seen with ADP8110 and ADP8117 cells in Fig. 5. Promoter activity was measured, and the averages of three independent trials are shown with their standard deviations.

ADP8081 (Δ*hcaB1*::*lacZ*-Km^r) and ADP8088 (Δ*hcaR1* Δ*hcaB1*::*lacZ*-Km^r). Since the *lacZ*-Km^r cassette contains the phage *fd* terminator (31), there should be minimal expression of genes downstream of the cassette, provided that the downstream genes are part of the operon into which the cassette is inserted. Due to this polar effect, strains ADP8081 and ADP8088 are effectively negative for the downstream *hcaC* gene. The constitutive level of LacZ expression found with strain ADP8088 is unmatched by comparable induction of LacZ in ADP8081 cells (Fig. 8), echoing the conclusion that activation of the hydroxycinnamate is required for induction.

Results on solidified minimal medium containing succinate, a hydroxycinnamate, and X-Gal served to confirm and extend the quantitative LacZ results. On plates containing succinate plus *p*-coumarate, ferulate, or caffeate at a concentration of 1 mM, strains ADP8110 (*hcaE*::*lacZ*-Km^r), ADP8114 (Δ*hcaA1* *hcaE*::*lacZ*-Km^r), and ADP8116 (Δ*hcaB3* *hcaE*::*lacZ*-Km^r) formed blue colonies, indicating cleavage of X-Gal by LacZ and thus expression of *hcaE*::*lacZ* off the *hca* promoter, whereas strain ADP8113 (Δ*hcaC1* *hcaE*::*lacZ*-Km^r) colonies appeared pale. Strains ADP8110, ADP8114, and ADP8116 were also tested on succinate medium containing caffeate, *p*-coumarate, or ferulate at 10^{-5} and 10^{-6} M. Only ADP8114 (Δ*hcaA1* *hcaE*::*lacZ*-Km^r) formed deep blue colonies on media containing the low concentrations of hydroxycinnamates. Thus, the data show conclusively that thioester intermediates are the true inducers of the operon that includes *hcaABCDE*.

Genes associated with the major *Acinetobacter hca* transcript. The major *hca* transcript is assumed to include *hcaA* and *hcaB*, given that they are separated by only 36 bp. As noted above, the *lacZ*-Km^r cassette used in this work contains a transcription terminator that should have a polar effect. Unlike *hcaB* deletion strain ADP8084, strain ADP8081 (Δ*hcaB*::*lacZ*-Km^r) and constitutive strain ADP8088 (Δ*hcaR1* Δ*hcaB*::*lacZ*-Km^r) fail to grow on *p*-coumarate. This phenotype can only be due to the absence of expression of HcaC in the latter strain, because, of all the genes between *hcaB* and *pobA* (Fig. 1B and 2), only *hcaC* is required for growth at the expense of hydroxycinnamates (55; Parke, unpublished). The result indicates that *hcaC* is part of the *hcaAB* transcript. Finally, polarity of the Δ*hcaC1* frameshift mutation demonstrated in strain ADP8136 (Δ*hcaC1* Δ*hcaR1* *hcaE*::*lacZ*-Km^r) (Fig. 8) compared to expression of *hcaE*::*lacZ* in ADP8117 (Δ*hcaR1* *hcaE*::*lacZ*-Km^r) (Fig. 6) indicates that *hcaC* and *hcaE* share a common transcript. Thus, at a minimum, the major *hca* transcript includes *hcaABCDE*.

DISCUSSION

Chromosome striding by means of an endogenous vector. Introduction of a vector into a chromosomal site that flanks DNA targeted for cloning is an effective and simple method for capturing DNA that neighbors a region already cloned (35). One advantage of this method over PCR for chromosome walking, aside from simplicity, yield, and fidelity to the wild-type sequence, is in the length of DNA that can be cloned. The insert of pZR8200 captured by this method was 16.5 kb. The stored lysate from the heterogenote *Acinetobacter* strain that contained the endogenous pUC vector for the cloning of pZR8200 was used to walk further down the chromosome. By this means, an insert was isolated that totaled 23 kb yet still maintained the functional *hca* genes that allowed *E. coli* to convert *p*-coumarate to protocatechuate (Parke, unpublished).

One potential outcome of vector insertional cloning, which is likely to occur with a strain in which the chosen vector can replicate, is the reisolation of the starting plasmid. The application of a selection for heterogenote formation (Fig. 1C) combined with a screen of *E. coli* colonies for the desired clones enabled this cloning method to work effectively.

Comparative organization of the genes for hydroxycinnamate dissimilation. In addition to having clustered genetic units that mediate related physiological functions, *Acinetobacter* sp. strain ADP1 has a remarkable tendency to exert unified control over related catabolic genes, exemplified by the *pcaIJFBDKCHG* operon (32). This trait continues with the *hcaABCDE* operon. The order of the *hcaAB* genes is similar to that of the homologous genes of *Pseudomonas* sp. strain HR199 (41, 48), *P. putida* WCS358 (59), and *P. fluorescens* AN103 (20), but a gene corresponding to *hcaC* does not appear to be linked to the other two in these strains. In *Amycolatopsis* sp. strain HR167 (1) and *S. paucimobilis* SYK-6 (34), a feruloyl-CoA synthetase gene lies immediately downstream of a gene homologous to *hcaA*. However, the ancestries of the latter ligase genes are remote from those of strains ADP1 or HR199. Organization of apparent *hca* gene homologs in the recently sequenced genome of *P. putida* KT2440 (25, 39) reveals an order similar to that of the *Acinetobacter hcaABC* genes. A divergently transcribed gene encodes a homolog of *hcaR*; nonetheless, the predicted product of this gene is remote

from HcaR, with only 29% of the aligned residues being identical.

The results presented here establish that the major *Acinetobacter hca* operon consists of at least *hcaABCDE*. Only 36 bp separate *hcaA* and *hcaB*, a transcription terminator in *hcaB* has a downstream effect on *hcaC*, and the Δ*hcaC1* frameshift mutation has a polar effect that extends to *hcaE* and possibly beyond it (Fig. 7). Sequence analysis of the previously isolated *Acinetobacter hcaCDEFG* genes revealed that *hcaF* represented a 390-bp ORF of unknown function (55). Because it is not known if *hcaF* is a functional ORF or part of an intergenic region between *hcaE* and *hcaG*, it is not yet clear whether the *hcaABCDE* operon extends beyond *hcaE*.

Identification of the thioester inducer of *hca genes*. Growth of *Acinetobacter* with caffeate, *p*-coumarate, and ferulate induces HcaG, chlorogenate esterase, whose level was monitored qualitatively by means of a T7-TAG marker (55). In a ligase-deficient strain, cells failed to grow at the expense of chlorogenate even though they were able to utilize quinate. An explanation for this observation was that a catabolite of caffeate may induce HcaG (55). This was the first suggestion that a hydroxycinnamate catabolite may trigger induction of an *hca* gene.

The more detailed analysis carried out with *hcaE*::*lacZ*-Km^r strain constructions for the present communication has pinpointed the inducing metabolite for the *hcaABCDE* promoter to be the thioester product of the hydroxycinnamate:CoASH ligase reaction. The results with HcaG (55) suggest that its gene may be governed in concert with the other *hca* genes, but it remains to be determined whether *hcaG* is part of the larger *hca* structural gene transcript. Although vanillin was observed to elicit expression of *vdh*, the vanillin dehydrogenase gene, in *Pseudomonas* sp. strain HR199 (48), the aldehyde had no inducing role in strain ADP1 (Fig. 6).

The simplest interpretation of the results with strain ADP8114 (Δ*hcaA hcaE*::*lacZ*-Km^r), in which induction was triggered by 10^{-7} M *p*-coumarate (Fig. 6), is that hydroxycinnamoyl-CoA interacts directly with HcaR. A few other regulatory molecules that appear to interact with aromatic thioesters have been identified. Catabolism of phenylacetic acid in *E. coli* and *P. putida* is carried out by the *paa* gene products through thioester formation, hydroxylation, and β-oxidation (17, 40). Repression of the *paaABCDEFGHIJK* operon mediated by PaaX is countered by the inducer phenylacetyl-CoA (18, 19). BadR, a MarR homolog atypical in being an activator, controls expression of genes involved in anaerobic benzoate dissimilation, and benzoyl-CoA may be its coinducer molecule (15).

HcaR, a novel member of the MarR family of regulators. MarR negatively regulates multiple antibiotic resistance and oxidative stress operons in *Salmonella* and *E. coli* (3). Diverse compounds relieve repression by the protein (53), and among these are aromatic compounds (57). Involved in regulating genes related to antibiotic resistance, the MarR homologs EmrR from *E. coli* (7, 33) and MexR from *Pseudomonas aeruginosa* (16, 47, 56) also appear to respond to specific aromatic compounds.

Within the highly divergent MarR evolutionary family are other regulators, in addition to BadR and HcaR, which govern aromatic catabolism in bacteria. CinR from *Butyrivibrio fibrisolvens* E14, which represses a cinnamoyl ester hydrolase gene, responds in vitro to sugars that contain ferulate (10). The repressor HpcR from *E. coli* strain C controls genes for catabolism of homoprotocatechuate (50). Although apparently not the primary regulator of the *cbaABC* genes for chlorobenzoate catabolism in *Comamonas testosteroni* BR60, CbaR, encoded by a divergently transcribed gene, responds to chlorobenzoate and may have a role in modulating expression (49). With respect to HcaR, hydroxycinnamates tend to inhibit cell growth at higher concentrations. Thus, the evolutionary relationship between MarR and a regulator like HcaR, which itself evolved to allow its host to cope with two demands, detoxification and nutrition, is intriguing.

In the context of the 56-kb *Acinetobacter* island of catabolic diversity, HcaR is an evolutionarily atypical regulator. The four regulators that govern the *pca* (21), *qui*, *pob* (14), and *dca* (Parke, unpublished) structural genes are all members of the small IclR family, and the two that govern the aromatic catabolic genes are known to be activators. Located elsewhere on the chromosome and required for catabolism of ferulate, the vanillate genes (52) are regulated by VanR, a repressor unrelated to HcaR (38).

Regulatory controls for hydroxycinnamate catabolic genes cloned from other bacteria have not been established. It will be interesting to learn whether the conservation observed in structural genes is echoed in common elements of regulatory control. To our knowledge, HcaR is a novel member of the MarR family in possessing the dual characteristics of being a repressor and recognizing an aromatic thioester. Given the apparently broad specificity of the hydroxycinnamate catabolic enzymes, product induction may well serve to enhance the specificity of induction, thus circumventing dissimilation of hydroxy- and methoxycinnamates that produce dead-end catabolites.

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