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Toxicity Caused by Hydroxycinnamoyl-Coenzyme A Thioester Accumulation in Mutants of *Acinetobacter* sp. Strain ADP1

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Hydroxycinnamates, aromatic compounds that play diverse roles in plants, are dissimilated by enzymes encoded by the *hca* genes in the nutritionally versatile, naturally transformable bacterium *Acinetobacter* sp. strain ADP1. A key step in the *hca*-encoded pathway is activation of the natural substrates caffeate, *p*-coumarate, and ferulate by an acyl:coenzyme A (acyl:CoA) ligase encoded by *hcaC*. As described in this paper, *Acinetobacter* cells with a knockout of the next enzyme in the pathway, hydroxycinnamoyl-CoA hydratase/lyase (HcaA), are extremely sensitive to the presence of the three natural hydroxycinnamate substrates; *Escherichia coli* cells carrying a subclone with the *hcaC* gene are hydroxycinnamate sensitive as well. When the *hcaA* mutation was combined with a mutation in the repressor HcaR, exposure of the doubly mutated *Acinetobacter* cells to caffeate, *p*-coumarate, or ferulate at 10^{-6} M totally inhibited the growth of cells. The toxicity of *p*-coumarate and ferulate to a Δ*hcaA* strain was found to be a bacteriostatic effect. Although not toxic to wild-type cells initially, the diphenolic caffeate was itself converted to a toxin over time in the absence of cells; the converted toxin was bactericidal. In an *Acinetobacter* strain blocked in *hcaA*, a secondary mutation in the ligase (HcaC) suppresses the toxic effect. Analysis of suppression due to the mutation of *hcaC* led to the development of a positive-selection strategy that targets mutations blocking HcaC. An *hcaC* mutation from one isolate was characterized and was found to result in the substitution of an amino acid that is conserved in a functionally characterized homolog of HcaC.

Aromatic compounds termed hydroxycinnamates are among potential carbon sources released from living and decaying plants. Consisting of variations on a phenolic ring with a propenoate side chain, hydroxycinnamates play versatile roles as components or precursors in plant architecture and defense (9, 48). The ability of bacteria to utilize diverse hydroxycinnamates as sources of carbon and energy is widely distributed among microbial groups (1, 8, 29, 32, 39, 46, 47). As is frequently the case with aromatic compounds (44), hydroxycinnamates tend to be toxic to bacterial cells (38). A bacterial strain that meets the challenge presented by these compounds is *Acinetobacter* sp. strain ADP1. However, the observation that one class of *Acinetobacter* mutants blocked in hydroxycinnamate catabolism was extremely sensitive to the presence of hydroxycinnamates led to an investigation of this phenomenon, and this investigation is reported here.

As a derivative of strain BD413 (23), *Acinetobacter* sp. strain ADP1 has a remarkable proficiency for natural transformation by DNA (30). Another notable feature of the strain is found in the extended regions of DNA where genes encoding catabolic functions are concentrated (20, 35). One cluster contains genes for pathways of dicarboxylate (*dca*), protocatechuate (*pca*), quinate or shikimate (*qui*), and *p*-hydroxybenzoate (*pob*) catabolism (35). Characterization of DNA beyond the *pob* genes in this strain led to the identification of all of the *hca* genes involved in hydroxycinnamate utilization (37, 46). The *pca*, *qui*, *pob*, and *hca* genes are functionally related in that quinate, shikimate, *p*-hydroxybenzoate, and hydroxycinnamates are all degraded via the protocatechuate branch of the β-ketoadipate pathway.

Enzymes encoded by the *hca* genes exhibit relatively broad substrate specificity, acting on three hydroxycinnamates, caffeate, *p*-coumarate, and ferulate, or their respective derivatives. In *Acinetobacter* strains, dissimilation of the latter two compounds occurs via the intermediates *p*-hydroxybenzoate and vanillate, respectively (Fig. 1) (8, 37, 46). The *hca* genetic cluster consists of two groups of genes: *hcaABCDEFG* and the divergently transcribed *hcaKR* (Fig. 2). Enzymes encoded by *hcaC, hcaA*, and *hcaB* carry out three steps in the dissimilation of hydroxycinnamates (Fig. 1). HcaC, hydroxycinnamoyl:CoA ligase, activates hydroxycinnamates to their thioester derivatives. HcaA, a bifunctional hydratase/lyase (13), converts the thioester derivative to an aldehyde intermediate. HcaB, an aldehyde dehydrogenase, transforms the aldehyde to a carboxylated derivative that serves as a substrate for downstream catabolic pathways. A fourth gene, *hcaG*, encodes an esterase that hydrolyzes chlorogenate to quinate and caffeate (46). An exception to the proximity of related genes is the situation for the *van* genes for vanillate degradation, which are unlinked to the *hca* region (43).

Accumulation of the substrate of PcaB, a tricarboxylate intermediate, is toxic to cells (14). Thus, upon their exposure to an appropriate precursor, *pcaB*-blocked cells give rise to secondary mutants that are protected by their inability to generate the tricarboxylate compound. The application of a positive-selection procedure based on the toxic effect has resulted in the isolation of informative structure-function mutations in upstream enzymes and their regulators (7, 14, 26, 27, 34). However, this approach had met with limited success in the case of the initial steps of the hydroxycinnamate pathway because the

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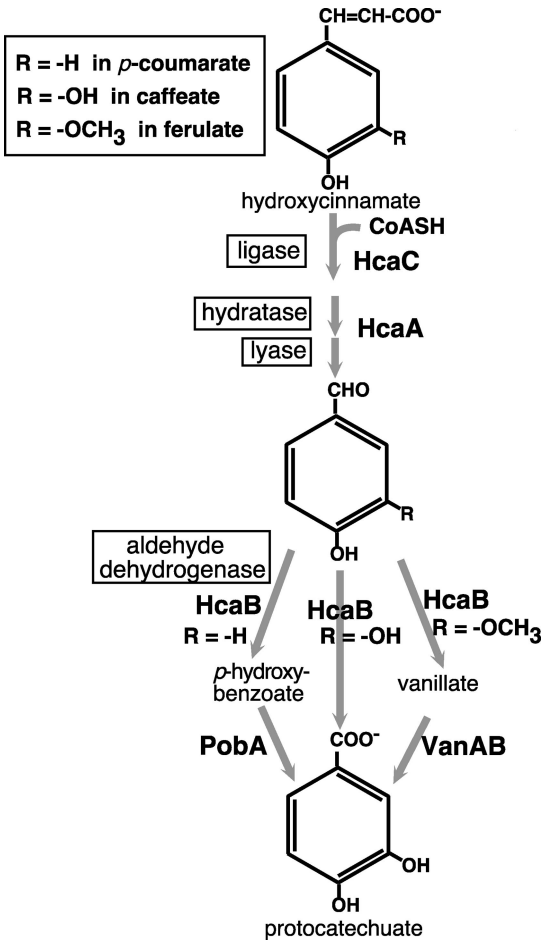

FIG. 1. Pathway for dissimilation of hydroxycinnamates to protocatechuate in *Acinetobacter* sp. strain ADP1.

numerically predominant secondary mutants were those blocked in steps towards the end of the catabolic sequence.

The gene responsible for controlling the expression of the *hca* genes, *hcaR*, encodes a repressor which is homologous to the *Escherichia coli* repressor, MarR, and the inductive triggers for *hca* gene expression were found to be the thioester products of the HcaC ligase reaction (37). The latter finding was based in part on results obtained with strain ADP8114, which contains two mutations: a promoterless *lacZ* fused to *hcaE*, and Δ*hcaA1*. In the presence of only 10^{-6} M *p*-coumarate, strain ADP8114 expressed LacZ at a level representing a 270-fold increase over the uninduced level, whereas other *hcaE*::*lacZ* strains with wild-type or *hca* mutant backgrounds had the background level of expression in the presence of low concentrations of hydroxycinnamates.

The impetus for the investigation reported in this paper was the observation of extreme hydroxycinnamate sensitivity in *hcaA* mutant strains like strain ADP8114. Experiments were designed to examine the nature of the poisoning, to define the sensitivity of *hcaA*-deficient cells to external hydroxycinnamates, to determine whether a knockout of *hcaR* increased such sensitivity, to discover whether the effect was bacteriostatic or bactericidal, and to explore whether the toxic effect could be developed as a method of positive selection targeted to mutations in hydroxycinnamate: CoA ligase.

MATERIALS AND METHODS

Media components. Luria-Bertani (LB) medium (45) or minimal medium (38) was used to grow cells. Sigma-Aldrich Co. was the source of hydroxy-*trans*-cinnamates and other growth substrates. Single substrates were succinate at 10 mM, quinate at 5 mM, and hydroxycinnamate (*p*-coumarate, ferulate, or caffeate) at 2 mM, with hydroxycinnamates being prepared from a 1 M stock solution in dimethyl sulfoxide (DMSO). Dual-substrate medium contained succinate at 10 mM and a hydroxycinnamate at a concentration that depended on the selection applied, as noted below; succinate used in liquid minimal medium was added prior to autoclaving. Pantothenate-supplemented minimal medium was tested by using the vitamin at 2 μg ml^{-1}. For antibiotic selections, LB medium was supplemented with ampicillin at a concentration of 100 μg ml^{-1} or with kanamycin at 15 μg ml^{-1} (for *Acinetobacter* cells) or 25 μg ml^{-1} (for *E. coli* cells). LB medium containing 5% sucrose was used to select against cells that carried a *sacB*-Km^r cassette.

Plasmid and strain construction. Table 1 lists strains and plasmids used in this study. Standard methods were used for molecular biology manipulations (3, 42). Cells of *Acinetobacter* were made naturally competent by published methods (22). Crude lysates were prepared by the resuspension of pelleted cells from a 5-ml culture in 500 μl of lysis buffer (22), followed by incubation at 60°C for 1 h; lysates of cells for PCRs were similarly prepared from 1 ml of a succinate overnight culture.

Strains with designed deletions were created by using intermediary strains containing a *sacB*-Km^r cassette from pRMJ1 in the targeted site. Cells having the cassette will not grow at room temperature in medium supplemented with 5% sucrose (21). Thus, the cassette affords positive selection for its replacement by DNA carrying a deletion. For example, strain ADP8095 was constructed from strain ADP853 by transformation of the latter with pZR8227, which contained a PstI deletion covering *hcaB* through *hcaK*; a *sacB*-Km^r cassette was inserted in the deletion site. Following selection and purification on medium supplemented with kanamycin, the resultant isolate ADP8090 was transformed with pZR8216, which contained Δ*hcaA1*. Colonies that grew in the presence of sucrose were screened for the absence of the cassette's Km^r marker as well as for the absence of vector antibiotic resistance. One of these colonies was isolated as strain ADP8095. The construction of other strains by following similar steps can be traced in Table 1. PCRs were performed to confirm that the desired insertion or deletion mutations were present in the *Acinetobacter* chromosome.

Acinetobacter growth tests. Comparative growth tests with succinate-supplemented medium were conducted with cultures grown at 37°C with shaking at 250 rpm. After overnight growth at the expense of succinate, a 100-μl aliquot of a culture was added to 5 ml of fresh minimal medium containing succinate with or without the test compounds; the initial cell concentration was about 10^7 cells ml^{-1}. The succinate control tubes also contained the amount of DMSO added with the highest level of hydroxycinnamate in a given experimental set. When cells exposed to succinate alone had reached the late exponential to early stationary phase (after about 4.5 h), turbidity was measured for all cultures of that strain. Pantothenate-supplemented medium was tested for strain ADP8085 grown in the presence of 10^{-5} M *p*-coumarate.

Absorbance of caffeate in minimal medium at 600 nm can interfere with the measurement of turbidity. This was not a problem with cells that can utilize caffeate readily. For cells with an *hcaA* or *hcaC* mutation, which are unable to utilize caffeate, the optical density at 600 nm (OD_{600}) of control tubes with caffeate at 10^{-3} or 10^{-4} M (without cells present) was subtracted from the values obtained with cells exposed to the respective concentrations of the compound. Similar results were obtained when cells were centrifuged from medium containing caffeate and resuspended in minimal medium. These results correlated well with apparent cell densities in other tubes containing known concentrations of cells.

***E. coli* growth tests.** Three independent isolates of *E. coli* strain DH5α freshly transformed with a ligation mix were cultured in liquid LB medium with ampicillin and yielded three separate preparations of pZR8266, carrying a full-length copy of *hcaC*. SphI deletion plasmids were prepared from each of the three pZR8266 stocks. The deletion plasmids, termed pZR8272, also had the full-length *hcaC* insert. A further ClaI-SmaI deletion of one pZR8272 preparation removed the 5′ half of *hcaC* along with insert DNA between the ClaI site and the vector multiple-cloning site, creating pZR8273.

E. coli cells were transformed with the three separate ligation mixes for the pZR8272 constructions as well as the ligation mix for the pZR8273 construction. Transformants that arose on LB plates containing ampicillin were patched onto

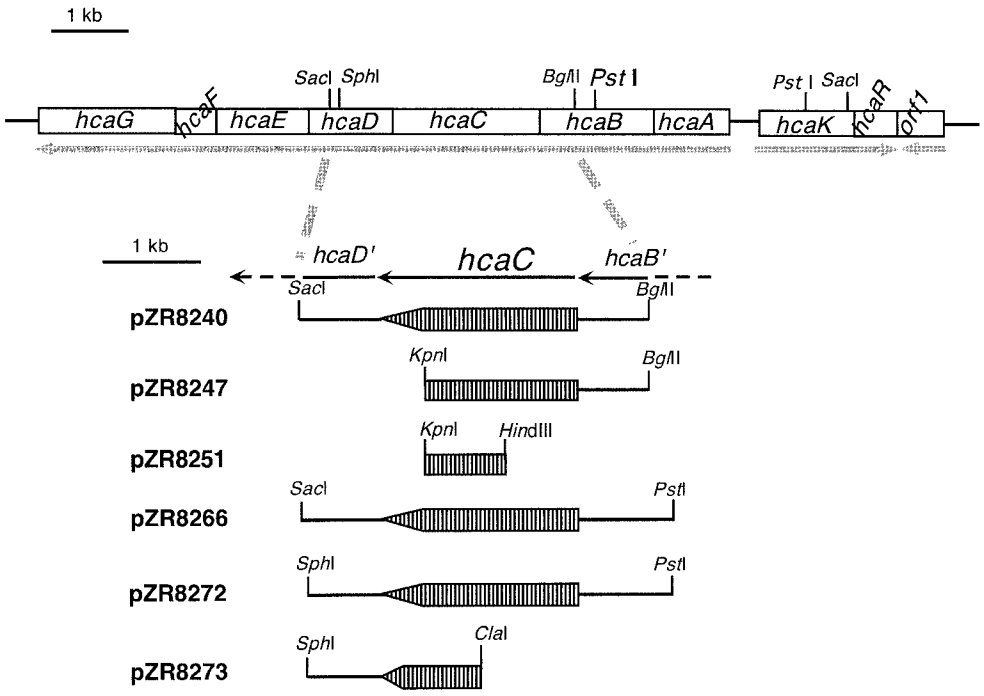

FIG. 2. Physical map of *hca* genes in *Acinetobacter* sp. strain ADP1. Arrows indicate directions of transcription but not necessarily the units of transcription; it has been established that at a minimum *hcaABCDE* forms a unit of transcription (37). The subclones shown below the map were used as transforming DNA to localize second-site suppressor mutations or in tests with *E. coli*.

the same medium alone and onto the same medium containing 1 mM *p*-coumarate; four colonies were patched for each independent construction.

Viability tests. To gauge the effect of hydroxycinnamoyl-coenzyme A (CoA) accumulation on cell viability, overnight cultures of cells grown on succinate were inoculated into medium containing 1 mM hydroxycinnamate and no other carbon source; the initial cell concentration was about 5×10^5 cells ml^{-1}. Control tubes with no carbon source added were amended with DMSO as noted above. Viable counts of cultures were determined after incubation for 48 h at 30°C with shaking at 250 rpm.

Isolation of *hcaC* secondary mutant strains. All transformations described in this section were carried out by spreading 150 μl of competent recipient cells on solidified nonselective medium containing succinate. Aliquots of DNA were placed on the cells at marked locations. Alternatively, when numerous mutations were mapped, cells were replica plated onto solidified medium that had been spread with 10 μl of a dilute DNA lysate of *E. coli* cells carrying a subclone. Following incubation at 37°C for 3 h, cells exposed to DNA were patched or replica plated onto selective medium along with control spots where no DNA or control DNA was added.

Figure 3 gives an overview of a procedure that was used to isolate *hcaC* mutants. Individual colonies of strain ADP8095 (*pcaB853* Δ*hcaA1*) were patched onto solidified medium containing succinate plus 2 mM *p*-coumarate (Fig. 3B). Colonies that appeared out of the background of nongrowing cells were purified on the same selective medium, and lysates were prepared. An aliquot of each crude lysate was treated with the protein precipitation solution of the Wizard genomic DNA purification kit (Promega Corp.) in order to ensure the full digestibility of the DNA. Further steps with the Wizard kit for purification of DNA from gram-negative bacteria, carried out with proportionately reduced volumes, were followed to completion. Digestion of the purified DNA with PstI physically separated *hcaC* from Δ*hcaA* (Fig. 2).

A subsequent selection required the use of competent cells of *pcaB* mutant strain ADP853 as recipients for the PstI-digested DNA of strains containing a putative *hcaC* mutation. The parental strain ADP8095 was constructed to contain the *pcaB853* mutation of strain ADP853 as well as the Δ*hcaA1* mutation. The strain was constructed in this way in order to avoid simply correcting the *pcaB* mutation of strain ADP853 rather than introducing the secondary mutation. The selective medium for plating the ADP853 transformants contained succinate and 0.1 mM *p*-coumarate. After purification of selected colonies on the same medium, the *pcaB* mutation in each strain was corrected by an analogous procedure. Transformation with pZR3, which carries *pcaB*, was followed by selection for growth at the expense of quinate, and strains were purified on the same medium (Fig. 3B).

Mapping of *hcaC* mutant strains. Resultant *p*-coumarate-negative strains were screened to verify that they did not carry pZR3 vector antibiotic resistance, that they contained a wild-type *hcaA* gene, and that the sole defect preventing their growth on hydroxycinnamates lay in *hcaC*. The last two conditions were established by testing the ability of pZR8240, which contains primarily the wild-type *hcaC* gene (Fig. 2), to correct the *p*-coumarate-negative phenotype. Mutations in strains that were found to have defects in *hcaC* were localized by means of other *hcaC* subclones (Fig. 2).

Frequency of second-site suppressor mutants. The frequency of second-site suppressor mutants of strains ADP8085 and ADP853 was determined by plating cells on selective medium containing 10 mM succinate plus *p*-coumarate at 0.1 and 2 mM, respectively. The fraction of the total viable count that appeared as CFU on the selective medium after 40 h at 37°C was measured as the second-site mutant frequency.

PCRs and DNA sequencing. Template DNA was prepared as a crude chromosomal lysate. A 2.3-kb amplicon of *hcaC* containing a spontaneous mutation was amplified by PCR. Conditions for PCR were 94°C for 3 min and 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. For each strain, reaction products were purified by a QIAquick PCR purification step (QIAGEN Inc.) prior to sequencing. On the basis of localization of spontaneous mutations, the relevant section of *hcaC* containing a spontaneous mutation was sequenced. Primer sets were as follows: HCACF1 (5′-TGTTCGCTTGGCAAATGACAGTGAGT-3′) and HCACR1 (5′-AACCCCATACGAAATGAGCTTGAGAA-3′) were used to amplify *hcaC*, HCACF3 (5′-CATCAGATTGCCAAGTTTTTATTTAC-3′) was used to sequence *hcaC2*, and DPZR82F6 (5′-GGGTCACACTTAATCGTCCT CACAA-3′) and DPZR82R3 (5′-TCTTCATTCTGATCCCATGTCAGCT-3′) were used to confirm the genotypes of strains ADP8093 and ADP8095.

ABI PRISM terminator cycle sequencing with AmpliTaq DNA polymerase was carried out at the Yale Keck Biotechnology Resource Lab.

RESULTS

Sensitivity of strain ADP8085 (Δ*hcaA1*) cells to hydroxycinnamates. A deletion in the hydratase/lyase gene, Δ*hcaA1*, elim-

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Genotype	Reference or source
E. coli strain			
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)		23
ADP853	*pcaB* frameshift mutation created by filling in the ends of an NcoI site	*pcaB853*	34
ADP1027	100-bp deletion in *hcaC*; blocked in hydroxycinnamate-CoASH ligase	Δ*hcaC1*	46
ADP8078	Km^r; Δ(*hcaRorf1*)*1*::*sacB*-Km^r in the ADP1 chromosome	Δ(*hcaR-orf1*)*1*::*sacB*-Km^r	37
ADP8079	Km^r; Δ(*hcaB′ AK′*)*1*::*sacB*-Km^r in the ADP1 chromosome	Δ(*hcaB′ AK′*)*1*::*sacB*-Km^r	37
ADP8083	217-bp HincII deletion in *hcaR* in the ADP8078 chromosome	Δ*hcaR1*	37
ADP8085	163-bp HpaI deletion in *hcaA* in the chromosome of ADP8079	Δ*hcaA1*	37
ADP8089	Km^r; Δ(*hcaB′ AK′*)*1*::*sacB*-Km^r from pZR8227 introduced into the ADP8083 chromosome	Δ*hcaR1* Δ(*hcaB′ AK′*)*1*::*sacB*-Km^r	This study
ADP8090	Km^r; Δ(*hcaB′ AK′*)::*sacB*-Km^r from pZR8227 introduced into the ADP853 chromosome	*pcaB853* Δ(*hcaB′ AK′*)::*sacB*-Km^r	This study
ADP8093	Δ*hcaA1* from pZR8216 introduced into the chromosome of ADP8089	Δ*hcaA1* Δ*hcaR1*	This study
ADP8095	Δ*hcaA1* from pZR8216 introduced into the chromosome of ADP8090	*pcaB853* Δ*hcaA1*	This study
ADP8150	Spontaneous *hcaC* mutation in ADP8095 mutant background	*pcaB853* Δ*hcaA1 hcaC2*	This study
ADP8209	Spontaneous *hcaC* mutation in wild-type background	*hcaC2*	This study
ADP8292	ADP8150 with *pcaB* mutation corrected with pZR3	Δ*hcaA1 hcaC2*	This study
Plasmids			
pBKS	Ap^r; narrow-host-range cloning vector		Stratagene
pBKSΔ1	Ap^r; pBKS (ΔSmaI-HincII)		37
pRMJ1	Ap^rKm^r; contains *sacB*-Km^r cassette for genetic replacement by positive selection		21
pRK415	Tc^r; broad-host-range vector		24
pUC18	Ap^r; narrow-host-range cloning vector		49
pZR3	Ap^r; 2.75-kb HindIII fragment containing *pcaB′DK′* from strain ADP1 in pUC18		10
pZR8200	Ap^r; 16.5-kb insert which includes the *hca* genetic cluster; in pUC19		37
pZR8208	Tc^r; 6.5-kb SacI insert of *hcaK′ABCD′* from pZR8200 in pRK415		This study
pZR8210	Ap^r; 6.5-kb SacI insert of pZR8200 in pBKSΔ1		37
pZR8216	Ap^r; 163-bp HpaI deletion of *hcaA* in pZR8210		37
pZR8221	Ap^r; 1.2-kb KpnI deletion of pZR8210; the insert contains *hcaABC′* and *hcaK′*		This study
pZR8227	Ap^r Km^r; *sacB*-Km^r cassette of pRMJ1 inserted at the site of the PstI deletion of *hcaB′ hcaA hcaK′* in pZR8220		37
pZR8240	Tc^r; 3.3-kb SacI-BglII insert of *hcaC* plus the 3′ part of *hcaB* in pRK415		37
pZR8247	Ap^r; 2.1-kb KpnI-BglII subclone of pZR8240 in pUC18		37
pZR8251	Ap^r; 1.3-kb HindIII deletion of pZR8247		This study
pZR8266	Ap^r; 3.4-kb PstI insert of *hcaB′CD′* from pZR8208 in pUC18		This study
pZR8272	Ap^r; 3.4-kb PstI-SphI insert of *hcaB′CD′* in pUC18		This study
pZR8273	Ap^r; pZR8272 with a 1.7-kb SmaI-to-ClaI deletion, leaving the 3′ end of *hcaC*		This study

inates the ability of *Acinetobacter* cells to grow at the expense of the three hydroxycinnamates (caffeate, *p*-coumarate, and ferulate) that are growth substrates for the wild-type strain (37). The mutation also blocks the cells' ability to grow in the presence of these compounds. The provision of wild-type *hcaA* by transformation of Δ*hcaA1* mutant strain ADP8085 with plasmid pZR8221 restored the hydroxycinnamate-positive phenotype.

To determine relative inhibitory concentrations of *p*-coumarate, caffeate, and ferulate, strain ADP8085 (Δ*hcaA*) and wild-type ADP1 cells at 10^7 cells ml^{-1} were exposed to decreasing concentrations of the three hydroxycinnamates in the presence of succinate (Fig. 4). Under these conditions, growth inhibition of strain ADP8085 was virtually complete at *p*-coumarate and caffeate concentrations of 10^{-5} M and higher and at a ferulate concentration of 10^{-3} M. Over the 4.5-h time period of the experiments, the growth of strain ADP1027 (Δ*hcaC1*) was similar to that of the wild-type strain in the presence of a 1 mM concentration of each of the three compounds (data not shown), indicating that the toxic effect observed under these

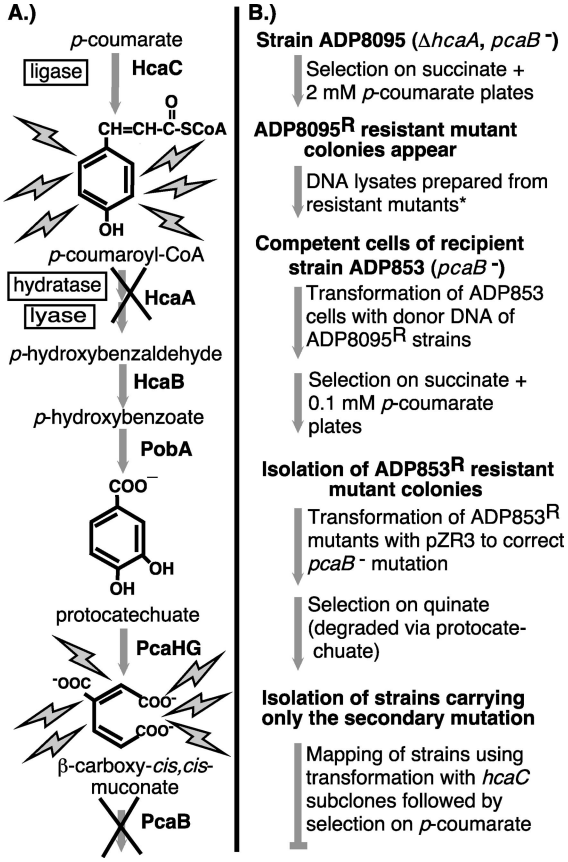

FIG. 3. Procedure for positive selection of strains whose sole impediment to growth with hydroxycinnamates is a mutation in HcaC, the hydroxycinnamate:CoA ligase. (A) Pathway for catabolism of *p*-coumarate to β-carboxy-*cis,cis*-muconate. A block at either of two steps, HcaA or PcaB, can result in a toxemia that prevents growth in the presence of precursor substrates. Mutation of an enzyme that acts upstream in generating the nonmetabolizable intermediate can overcome the inhibitory effect. (B) Flowchart showing the steps taken to isolate strains having a single mutation which lies in a gene other than *hcaA* or *pcaB*. The manipulations shown in lightface text were applied to the *Acinetobacter* strain in boldface text that headlines the steps that follow. The asterisk indicates that DNA prepared from the ADP8095 resistant mutants was digested with PstI to separate the *hcaA* deletion from secondary mutations, such as those in *hcaC*, which can block the conversion of *p*-coumarate to growth-inhibiting compounds. The relevant PstI site between *hcaA* and *hcaC* is shown in Fig. 2. Although the procedure for strain isolation seems elaborate, it is efficient because of the ability to process multiple strains readily due to the natural transformability of the strains and the ease of doing multiple transformations on a single plate.

conditions was not simply the result of the hydroxycinnamates being converted to toxins in the absence of metabolism.

The relative sensitivity of *pcaB* mutant strain ADP853 to *p*-hydroxybenzoate and to *p*-coumarate was determined by the same method. In this case, growth inhibition was virtually total in the presence of each of the two compounds at 10^{-4} M (data not shown). Thus, the toxic effect of *p*-coumarate on an *hcaA* mutant strain appears to be somewhat more potent than the effect of this compound on the carboxymuconate-accumulating *pcaB* mutant strain ADP853.

It should be noted that the toxic effect is dependent on the cell concentration. Thus, a 10-fold-lower initial cell density resulted in the total absence of the growth of strain ADP8085 cells in the presence of 10^{-6} M *p*-coumarate by the time the control cells on succinate alone had fully grown (data not shown).

Sensitivity of strain ADP8093 (Δ*hcaA1* Δ*hcaR1*) cells to hydroxycinnamates. A possible interpretation of the hydroxycinnamate toxic effect observed with Δ*hcaA1* mutant strain ADP8085 is that elevated expression of *hca* genes, triggered by the accumulation of hydroxycinnamoyl-CoA thioester inducers (37), places a deleterious biosynthetic load on cells. This interpretation was tested by deleting the HcaR repressor, thereby ensuring uniform *hca* gene expression in the derived strain in the presence of different concentrations of hydroxycinnamates and eliminating induction as a factor (37).

Strain ADP8093 (Δ*hcaA1* Δ*hcaR1*) was grown in the presence of different levels of hydroxycinnamates in parallel with its parental strain ADP8083 (Δ*hcaR1*). As shown in Fig. 5, strain ADP8083 grew well in the presence of hydroxycinnamates, whereas sensitivity to hydroxycinnamates was higher in strain ADP8093 than in strain ADP8085 (Δ*hcaA1*). When the concentration of *p*-coumarate, caffeate, or ferulate was lowered to 10^{-7} M, cultures of strain ADP8093 were able to grow somewhat (Fig. 5).

Rescue of an *hcaA* mutant from hydroxycinnamate toxicity by a second-site mutation. As explained in the section dealing with *hcaC* mutant isolation below, suppressor mutants were isolated, and the secondary mutations that rescued *hcaA* mutant cells from hydroxycinnamate poisoning were localized to *hcaC*. When strain ADP8292 (*hcaC2* Δ*hcaA1*) was exposed to any of the three hydroxycinnamates at a concentration of 1 mM, it grew at the expense of succinate as well as wild-type cells, unlike Δ*hcaA1* strain ADP8085 (data not shown). The rescue of Δ*hcaA1* cells by an *hcaC* mutation is consistent with the conclusion that hydroxycinnamoyl-CoA thioester accumulation inhibits the growth of an *hcaA* mutant strain in the presence of hydroxycinnamates. This effect could be attributed to the depletion of CoA pools by the sequestering of the CoA as the hydroxycinnamoyl-CoA thioester. To determine whether this postulated metabolic imbalance could be overcome by supplying pantothenate, a precursor of CoA, the vitamin was added to medium containing succinate plus 10^{-5} M *p*-coumarate. The pantothenate supplement did nothing to enhance the growth of strain ADP8085 (data not shown).

Effect of *p*-coumarate on *E. coli* cells carrying *hcaC*. Further evidence that supports hydroxycinnamoyl-CoA thioester buildup as the cause of growth inhibition in an *hcaA* mutant strain comes from results obtained with *E. coli* cells. The presence or absence of hydroxycinnamate should not influence regulation in this case. *E. coli*(pZR8272) cells contain a full-length *hcaC* expressed off the vector *lac* promoter, and *E. coli*(pZR8273) cells carry only half of *hcaC* (Fig. 2). When freshly plated, independent transformants of *E. coli*(pZR8272) cells were subcultured onto antibiotic medium with and without 1 mM *p*-coumarate, they grew only in the absence of the hydroxycinnamate, whereas cells of *E. coli*(pZR8273), like other isolates without an intact copy of *hcaC*, grew on both types of media.

Survival of Δ*hcaA* cells following exposure to *p*-coumarate or ferulate. To determine whether the effect of accumulated

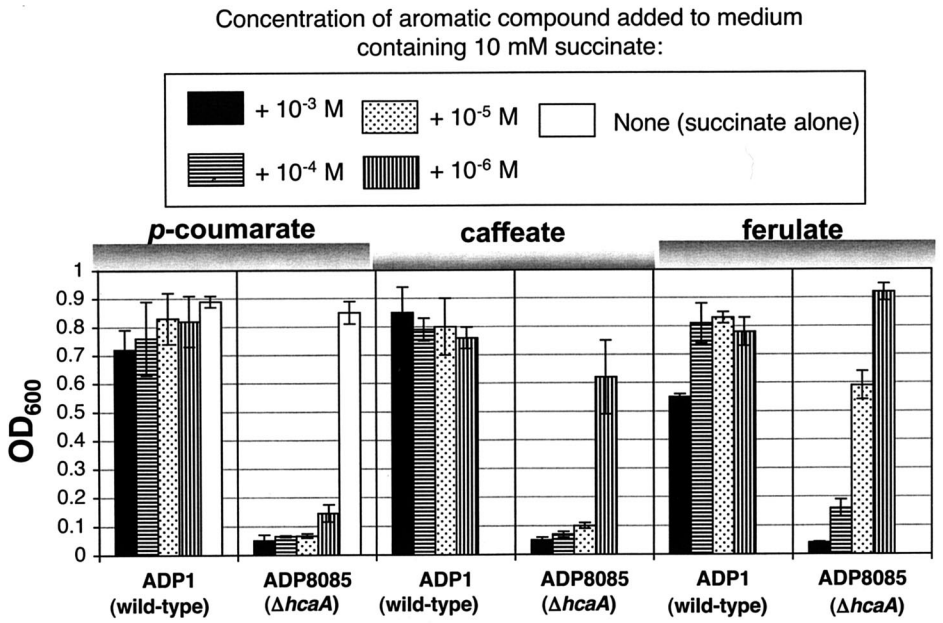

FIG. 4. Effects of hydroxycinnamates on wild-type strain ADP1 and Δ*hcaA1* strain ADP8085. Cells were grown at the expense of succinate with decreasing amounts of the three hydroxycinnamates added. For each set of data, OD_{600} was measured when succinate-grown cells had reached the late exponential to early stationary phase. The initial cell concentration was 10^7 cells ml^{-1}, which corresponded to an OD_{600} of 0.03. The averages for three independent trials are shown with their standard deviations (error bars).

thioesters is bacteriostatic or bactericidal, succinate-grown cells were inoculated at a low concentration into minimal medium containing only *p*-coumarate or ferulate at 1 mM. After incubation for 48 h at 30°C, cells were diluted and plated to measure viability. As shown in Fig. 6, cells exposed to the two compounds retained their viability over the 48-h period. An analogous test with *pcaB* mutant ADP853 exposed to *p*-hydroxybenzoate generated similar results.

Effect of caffeate on cell survival. One method for measuring substrate toxicity qualitatively is to supply a compound as a localized spot on a plate in or on which cells are distributed uniformly so that the substrate forms a concentration gradient (38). Examined in this way, caffeate appears to be nontoxic to strain ADP1 cells. However, the incubation of strain ADP8085 and other *Acinetobacter hca* mutant strains with 1 mM caffeate as was done with the other two compounds resulted in a loss of viability (Fig. 6). Because this result occurred with Δ*hcaC1* mutant strain ADP1027 as well, it was not possible to draw a conclusion about the mechanism of poisoning caused by the buildup of caffeoyl-CoA thioester.

In further tests of caffeate toxicity, the compound was incubated at a concentration of 1 mM in minimal medium at 37°C for 48 h and, thus prepared, was termed "converted caffeate." The brown converted caffeate was toxic to wild-type *Acinetobacter* cells. In medium containing converted caffeate, viable-cell numbers decreased by about 1 log unit after 2 h of incubation and by another log unit at 3 h, and viability was undetectable (less than 5 CFU ml^{-1}) by 5 h, at which time the viable-cell concentration in the control tubes containing only the added DMSO was 5×10^5 CFU ml^{-1} (standard deviation, 2×10^5 CFU ml^{-1}). The killing effect was reduced upon the lowering of the concentration of converted caffeate or the raising of the cell concentration, each by an order of magnitude.

Isolation of cells with spontaneous mutations in *hcaC*. As described in Materials and Methods and outlined in Fig. 3B, resistant mutants derived from strain ADP8095 were selected on solidified medium containing succinate plus 2 mM *p*-coumarate. *p*-Coumarate was used as the selective hydroxycinnamate due to the chemical instability of caffeate and the genetic instability of the *van* region of the ADP1 chromosome, required for the catabolism of ferulate (43). At this point, each resistant mutant of ADP8095 contained three mutations: *pcaB853*, Δ*hcaA*, and an unknown secondary mutation, predicted to be in *hcaC*.

Chromosomal lysates from the resistant mutants were digested with PstI in order to physically unlink Δ*hcaA* from *hcaC* (Fig. 2), with the goal being to obtain a strain having a mutation in the latter gene but not the former. Following the transformation of *pcaB*-negative strain ADP853 with PstI-digested DNA containing the putative *hcaC* mutation, cells were selected on succinate plus *p*-coumarate. Because the transforming DNA contained the *pcaB853* mutation, protection from toxicity was conferred by the putative *hcaC* mutation and not by the introduction of a wild-type *pcaB* gene. In order for the *hcaC* mutation to confer resistance to *p*-coumarate in the *pcaB*-negative background, it was necessary to lower the *p*-coumarate concentration in solidified medium to 0.1 mM; the rationale for lowering the concentration is discussed in the next section. Correction of the *pcaB* mutation by an *E. coli*(pZR3) lysate carrying the wild-type *pcaB* allele yielded strains that carried only the acquired *hcaC* mutations.

Frequency and types of suppressor mutants arising in an *hcaA* or *pcaB* mutant background at different concentrations

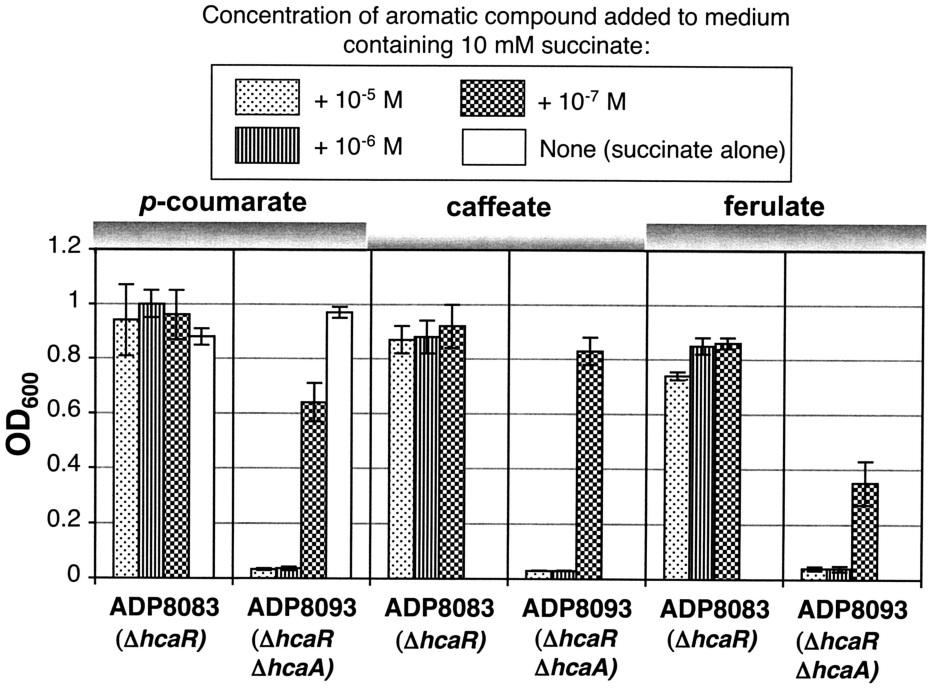

FIG. 5. Effects of hydroxycinnamates on Δ*hcaR1* strain ADP8083 and Δ*hcaA1* Δ*hcaR1* strain ADP8093. Cells were grown at the expense of succinate with decreasing amounts of the three hydroxycinnamates added, and they were measured as described in the legend to Fig. 4. The initial cell concentration was 10^7 cells ml^{-1}, which corresponded to an OD_{600} of 0.03. The averages for three independent trials are shown with their standard deviations (error bars).

of *p*-coumarate. The effect of *p*-coumarate concentration on the selection of suppressor mutations in *hcaA* strains versus that in *pcaB* mutant strains was investigated further. The frequency of suppressor mutants was determined for two strains, ADP8085 (*hcaA1*) and ADP853 (*pcaB853*). On succinate plus 2 mM *p*-coumarate, the suppressor mutant frequency was 1.8×10^{-6} for strain ADP8085 and 7.5×10^{-6} for strain ADP853. On succinate plus 0.1 mM *p*-coumarate, the second-site mutant frequency was 3.8×10^{-6} for strain ADP8085 and 11×10^{-6} for strain ADP853. It should be noted that the effective cell density was lower under the conditions of these experiments than under the conditions associated with the patch method described above, allowing a sufficient stringency of selection of suppressor mutations from strain ADP8085. The results for suppressor mutant frequencies for strains ADP8085 (*hcaA1*) and ADP853 (*pcaB853*) are consistent with the presence of more suppressor targets upstream of the *pcaB* gene product and with 2 mM *p*-coumarate providing slightly more stringent selective conditions, but they do not fully explain why a lower *p*-coumarate concentration is required to obtain *hca* suppressor mutants from strain ADP853.

p-Hydroxybenzoate is an intermediate in the catabolism of *p*-coumarate (Fig. 1). Should protection from accumulation of carboxymuconate in a *pcaB* mutant be afforded by a mutation in *pobA*, it is conceivable that *p*-hydroxybenzoate, accumulated by the *pcaB pobA* cells, could spill out into the medium and affect the growth of *pcaB* cells that are not similarly protected; an analogous situation would apply to an upstream block in *pcaH* or *pcaG*. These effects account for the results described in the next paragraph.

On media containing succinate plus 2 mM *p*-coumarate, virtually all of the secondary mutants of strain ADP853 (*pcaB853*) were resistant to 1 mM *p*-hydroxybenzoate as well, i.e., they were blocked in their metabolism of *p*-hydroxybenzoate, whereas on medium containing succinate plus 0.1 mM *p*-coumarate, about 70% of the ADP853 mutants were resis-

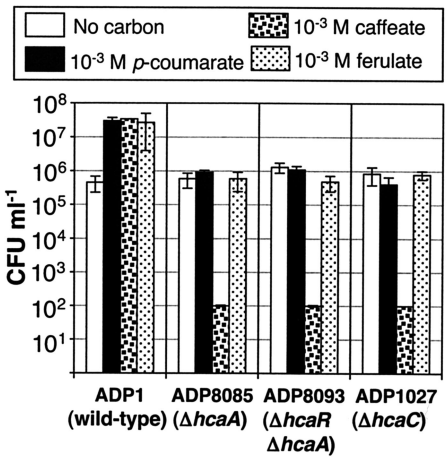

FIG. 6. Survival of *Acinetobacter* cells incubated in the presence of a hydroxycinnamate at 1 mM with no other substrate present. Viable counts are shown on a logarithmic scale. Cultures were maintained at 30°C for 48 h. The number of cells in caffeate was at the limit of detection and is actually smaller than the number indicated by the bar. Viable counts are means for three independent experiments, with error bars showing the standard deviations.

tant to *p*-hydroxybenzoate. Of the ADP853 secondary mutants that were sensitive to 1 mM *p*-hydroxybenzoate, i.e., putative *hca* mutants, all were resistant to *p*-coumarate at a level of 0.1 mM. However, in the presence of 2 mM *p*-coumarate, the *p*-hydroxybenzoate-sensitive putative *hcaC* mutants grew only if *p*-hydroxybenzoate-resistant cells were excluded from the plate. It is likely that *p*-hydroxybenzoate-resistant cells cause the accumulation of *p*-hydroxybenzoate or protocatechuate in the surrounding medium, which prevents the growth of strains that have acquired *p*-coumarate resistance through an *hcaC* mutation.

Sequence analysis of a representative HcaC mutation. Putative *hcaC*-mutated isolates that now had wild-type *hcaA* and *pcaB* alleles were screened to eliminate those that carried a deletion that extended beyond *hcaC*. Plasmid pZR8240, which carries *hcaC* (Fig. 2), transformed to wild type those cells for which an *hcaC* mutation was the sole barrier to growth with hydroxycinnamates. Subsequent mapping of the mutations was performed by testing the ability of the *hcaC* subclones (Fig. 2) to transform each mutant strain to a *p*-coumarate-positive phenotype, and sequencing confirmed the mapping results. Ten *hcaC* spontaneous mutant strains were isolated from the two-step ADP8095-ADP853 procedure. One of the mutations, carried by strain ADP8209, was a thymine-to-cytosine transition positioned 784 bp from the 5′ end of *hcaC*, altering a tryptophan codon to one for arginine at residue 262 in HcaC. The other *hcaC* mutations will be presented as part of a separate paper.

DISCUSSION

Sensitivity of cells to a block in hydroxycinnamoyl-CoA thioester catabolism. A block in the catabolism of hydroxycinnamoyl-CoA thioesters makes *Acinetobacter* cells sensitive to micromolar concentrations of hydroxycinnamates, a response that is enhanced in a constitutive mutant strain. This susceptibility is complemented by the ability of *hcaA* mutant cells containing a reporter *lacZ* gene to detect micromolar levels of hydroxycinnamates and to respond with a high level of induced expression (37). Cells with these characteristics have potential as environmental sleuths to explore the bioavailability of hydroxycinnamates in natural habitats where they may be bound to inert materials and to explore the hypothesis that one source of hydroxycinnamates for *Acinetobacter* sp. strain ADP1 is the plant matrix suberin, in which they are esterified to dicarboxylic acids (4, 15, 31). This hypothesis has particular resonance because in this strain many *dca* genes for the catabolism of dicarboxylic acids are just upstream of the *pca* structural genes (36) and thereby linked to the *hca* genes.

The elimination of hydroxycinnamate toxicity in an *hcaA* mutant strain by an *hcaC* mutation points to acyl-CoA thioester accumulation as a trigger for the toxic effect, as does the sensitivity of *hcaC*-carrying *E. coli* cells to *p*-coumarate. The effect of accumulated *p*-coumaroyl-CoA or feruloyl-CoA thioesters on cells was bacteriostatic, but that of the caffeoyl-CoA thioester could not be assessed because caffeate itself became bactericidal during the course of the experiment, having undergone oxidative browning (6). Oxidation of caffeic acid to the reactive *o*-quinone and to condensation products has been observed in simple, abiotic model systems (5, 12, 17, 25).

It remains unclear why accumulated hydroxycinnamoyl-CoA thioesters are growth inhibitory. Sequestering of CoA and interference of the accumulated thioesters with cellular processes are two reasonable hypotheses. In *E. coli*, reversible growth stasis occurred when the total CoA concentration fell below 5 pmol per 10^8 cells (19). In an effort to influence the intracellular CoA pool, *Acinetobacter* Δ*hcaA* mutant cultures were supplemented with the CoA precursor pantothenate (18), but this step failed to alleviate the growth stasis caused by the presence of *p*-coumarate. Thus, the cause of growth inhibition by hydroxycinnamoyl-CoA accumulation remains unresolved.

Knockouts of HcaA homologs were created in *Pseudomonas* sp. strain HR199, *Pseudomonas putida* WCS358, *Pseudomonas fluorescens*, and *Sphingomonas paucimobilis*, but toxic effects resulting from the block were not reported (13, 29, 33, 47). In the case of *S. paucimobilis*, the HcaA homolog, FerB, had an isofunctional protein that could fill in for its activity (29), but a knockout of enoyl-CoA hydratase/lyase in each of the other species resulted in a ferulate-negative phenotype. It may be that the conditions used for the mutant screens and mutant analysis were not suitable for detecting a ferulate-inhibitory phenotype.

Substrate concentration as a critical parameter in positive-selection schemes targeting genes of aromatic catabolic pathways. The frequency of second-site suppressor mutations was three- to fourfold higher in strain ADP853 (*pcaB* mutant) than in strain ADP8085 (*hcaA* mutant). An explanation for this result is that suppressor mutations of strain ADP8085 largely target *hcaC* but that those of strain ADP853 may include *pobA*, *pobR*, and *pcaHG* and, in the case of the low concentration of *p*-coumarate, *pcaU* and *hcaC* as well. At the lower *p*-coumarate concentration, the increase in suppressor mutation frequency is consistent with the emergence of additional colonies with more subtle defects that can protect cells from the selective condition; in addition, as noted above, there are more target sites for resistance in strain ADP853 at the lower concentration.

Positive selection for mutations in enzymes, such as protocatechuate 3,4-dioxygenase, that act upstream of the enzyme encoded by *pcaB* has generally used a Δ*pcaBDK* mutant exposed to substrate concentrations in the millimolar range (7, 14). In the present study, positive selection targeting mutations in HcaC carried out in an *hcaA* or an *hcaA pcaB* mutant background required a *p*-coumarate concentration above 1 mM in order to avoid background growth of cells. This condition was required with strain ADP8095 (*pcaB853* Δ*hcaA1*) cells from a single colony patched onto selective medium, due to the high concentration of cells in a given patch. It may seem counterintuitive that, following transformation with *hcaC*-mutated DNA, selection for HcaC mutants in a background mutated only in *pcaB* required that the *p*-coumarate concentration be dropped to 0.1 mM, particularly since strain ADP853 is less sensitive to the presence of *p*-coumarate than strain ADP8085, as noted in Results.

The problem appears to have been caused by feeding from non-*hca* mutants, e.g., *pob* or *pcaHG* mutants, in the population under selection. In the case of *pob* mutants, the level of *p*-hydroxybenzoate that accumulated in the medium was presumably sufficient to exert a separate selective pressure for

second-site suppressor mutations in genes required for the conversion of *p*-hydroxybenzoate to carboxymuconate.

Positive selection for mutations targeting hydroxycinnamate: CoA ligase. Ligases that convert hydroxycinnamates to their corresponding CoA thioesters belong to a biotechnologically significant group of enzymes. In the biotransformation of aromatic feedstocks into natural aroma chemicals (16, 40, 41), the microbial ligase is a key catalyst, and 4-coumarate:CoA ligase from plants produces hydroxycinnamoyl-CoA substrates that are central to the biosynthesis of specialized plant phenylpropanoids. The evolutionary lineage of HcaC is similar to that of the homologous Fcs, feruloyl-CoA synthetases, from *Pseudomonas* sp. strain HR199 (33) and *P. putida* strain KT2440 (38a); to our knowledge, other close homologs pulled up by a BLAST search (2) are uncharacterized at present. *Acinetobacter* HcaC and *Pseudomonas* Fcs proteins are divergent from functionally similar ligases from *Amycolatopsis* (1) and *Sphingomonas* (29) as well as from the two major classes of angiosperm 4-coumarate:CoA ligases (11, 28).

In principle, it should be possible to apply positive selection against the accumulation of hydroxycinnamoyl-CoA thioesters to isolate random or site-directed mutations in hydroxycinnamate:CoA ligases of diverse origins or specificities. In this study, one suppressor mutant isolated by the two-step ADP8095-ADP853 selection method contained a missense mutation that translates to the replacement of tryptophan with arginine at residue 262 of HcaC. Trp262 is conserved in the aligned Fcs homologs from *Pseudomonas* sp. strain HR199 (33) and *P. putida* strain KT2440 (38a).

It is likely that the accumulation of hydroxycinnamoyl-CoA thioesters is inhibitory to organisms in general, and the fact that their generation from hydroxycinnamates in *E. coli* cells carrying an *hcaC* subclone inhibits cell growth presents a potential opportunity for the selection of mutations in other hydroxycinnamate:CoA ligases, be they homologous to or divergent from HcaC. This fact also affords an easy preliminary functional genomics screen of putative ligase genes for which the aligned products are similar to characterized hydroxycinnamate:CoA ligases. Finally, the use of *hcaC* or a similar gene in a positive-selection cassette analogous to the *sacB*-Km^r construction (21) is under investigation.

Two methods for isolating *hcaC* mutants were described in this paper. Both of these methods led to the isolation of diverse HcaC mutations, which will be described in a separate paper. The two-step ADP8095-ADP853 procedure (Fig. 3B) that resulted in the isolation of the mutant strain ADP8209 has the advantage of homing in on *hcaC* specifically. The alternative, direct selection of strain ADP853 in the presence of a low concentration of *p*-coumarate, has the advantage of one less step but the disadvantage of yielding a high percentage of mutants that are blocked in steps downstream of the hydroxycinnamate pathway. The two methods for isolating *hcaC* mutant strains spotlight one application afforded by the discovery of hydroxycinnamoyl-CoA thioester poisoning, and it should now be straightforward to use PCR-mediated mutagenesis coupled with natural transformation (26, 27) to further target *hcaC* and to enrich for mutations that may be relevant to structure-function analysis of the ligase.

Strategies to deal with a toxic intermediate. Evidence presented in this paper points to the importance of cell concentration in the evolution of potentially poisonous pathways. Given the toxigenic nature of nonmetabolizable hydroxycinnamoyl-CoA thioesters and the possible toxicity of aldehyde intermediates, patchwork evolution of the pathway of hydroxycinnamate dissimilation found in bacteria like *Acinetobacter* may have presented special challenges. It is reasonable to hypothesize that the acquisition and expression of *hcaC* in the absence of *hcaA* would be selected against except with very low concentrations of hydroxycinnamates or at high cell concentrations, conditions that apply when cells are present in a biofilm, a condition that also facilitates gene transfer. Conversely, the evolution of catabolic pathways that traffic in toxic intermediates likely imparts a special selective value for biofilm formation.

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