Disruption of phacA, an Aspergillus nidulans Gene Encoding a Novel Cytochrome P450 Monooxygenase Catalyzing Phenylacetate 2-Hydroxylation, Results in Penicillin Overproduction*

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Aspergillus nidulans utilizes phenylacetate as a carbon source via homogentisate, which is degraded to fumarate and acetoacetate. Mutational evidence strongly suggested that phenylacetate is converted to homogentisate through two sequential hydroxylation reactions in positions 2 and 5 of the aromatic ring. Using cDNA substrauction techniques, we have characterized a gene, denoted phacA, whose transcription is strongly induced by phenylacetate and which putatively encodes a cytochrome P450 protein. A disrupted phacA strain does not grow on phenylacetate but grows on 2,5-dihydroxyphenylacetate. Microsomal extracts of the disrupted strain are deficient in the NADPH-dependent conversion of phenylacetate to 2-hydroxyphenylacetate. We conclude that PhacA catalyzes the ortho-hydroxylation of phenylacetate, the first step of A. nidulans phenylacetate catabolism. The involvement of a P450 enzyme in the ortho-hydroxylation of a monoaromatic compound has no precedent. In addition, PhacA shows substantial sequence divergence with known cytochromes P450 and defines a new family of these enzymes, suggesting that saprophytic fungi may represent a source of novel cytochromes P450.

Phenylacetate is a precursor for benzylpenicillin production. phacA disruption increases penicillin production 3–5-fold, indicating that catabolism competes with antibiotic biosynthesis for phenylacetate and strongly suggesting strategies for Penicillium chrysogenum strain improvement by reverse genetics.

Aerobic degradation of aromatic hydrocarbons by microbes involves the action of oxygenases (enzymes that incorporate one or two atoms from dioxygen into substrates) acting at two different levels in specific catabolic pathways (1, 2). First, oxygenase enzymes acting at the upstream segment of these pathways incorporate one (monooxygenases, aromatic ring hydroxylases) or two (aromatic ring dioxygenases) oxygen atoms into the aromatic substrate as hydroxyl groups, preparing the ring for a subsequent ring-opening step. In this second step, the dihydroxylated aromatic ring is opened by ring-cleavage dioxygenases.

Monooxygenases are a mechanistically diverse group of enzymes (1) including, for example, flavoproteins such as p-hydroxybenzoate hydroxylase (3), multicomponent enzymes such as Pseudomonas mendocina toluene 4-monooxygenase, in which one of the terminal hydroxylase polypeptides contains a binuclear iron cluster (4), or heme-containing cytochrome P450 systems. Monooxygenases of the cytochrome P450 superfamilly (5, 6) are widely distributed among living organisms and catalyze a multiplicity of biosynthetic and catabolic reactions, usually with narrow substrate specificity, including the hydroxylation of a variety of lipophilic drugs.

In common with other saprophytic microbes, the genetically amenable, obligate aerobic fungus Aspergillus nidulans shows notable metabolic versatility. For example, it can use the aromatic hydrocarbon compound phenylacetate (PhAc) as sole carbon source. Despite the abundant information available on the catabolic pathways of other aromatic compounds, which have been extensively studied in bacteria, our understanding of PhAc degradation pathways is scarce. In Pseudomonas putida U, it is known that PhAc is degraded through phenylacetyl-CoA (7), although the ring cleavage steps remain uncharacterized. In A. nidulans, PhAc degradation proceeds through 2,5-di hy droxy-PhAc (homogentisate, see Fig. 1). The three structural genes mediating the conversion of homogentisate to Krebs cycle intermediates (i.e. the “lower” PhAc pathway) have been characterized (8–10), but the steps leading to homogentisate have not yet been reported. We describe here mutational and molecular analysis showing that A. nidulans PhAc catabolism proceeds via homogentisate through two sequential hydroxylation steps, of which the first is the 2-hydroxylation of the ring catalyzed by a novel cytochrome P450. Targeted inactivation of this gene results in penicillin overproduction.

EXPERIMENTAL PROCEDURES

Fungal Strains, Media, and Growth Conditions—A nidulans strains carried markers in standard use (11). Standard media for A. nidulans (12) were used for strain maintenance, growth tests, and transformation. Complementation tests were carried out in constructed diploids. A bioA1 strain was the source of cDNA, and a bioA1 methG1 argB2 strain was the recipient strain for phacA gene disruption. A bioA1 methG1 strain was used as wild type control in experiments with the disrupted strains. Culture conditions inducing high levels of expression of the PhAc catabolic genes have been described (13) and were used to grow mycelia for protein extraction. PhAc and its monohydroxy and dihydroxy derivatives were used as sole carbon source at 10 mM (although homogentisate was occasionally used at 25–50 mM), and 10 mM ammonium chloride was used as sole nitrogen source.

* This work was supported by Spanish Comisión Interministerial de Ciencia y Tecnologı´a Grants BIO94-932 and BIO97-348 and Antibiotı´co Investigaciones Cientı´ficas, Vela´zquez 144, Madrid, 28006, Spain

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The abbreviations used are: PhAc, phenylacetate; CYP, cytochrome P450; kbp, kilobase pair(s); HPLC, high performance liquid chromatography.

This paper is available on line at http://www.jbc.org
Isolation of PhAc Nonutilizing Mutants—2-, 3-, and 4-fluorophenylacetate at a 5 mM concentration were shown to prevent growth of *A. nidulans* in the presence of a derepressing carbon source, indicating that their catabolism was toxic for the mold. However, some residual growth was observed when plates were incubated for more than 3 days at 37 °C. Sectors of markedly more vigorous mycelia formed after prolonged incubation. Although the reason why catabolism of these PhAc derivatives results in toxicity is not clear, preliminary tests showed that mutations preventing the toxicity of 2- and 3-fluorophenylacetate also prevented the catabolism of PhAc. Therefore, conidiospores of a *A. nidulans* strain were plated to obtain isolated colonies, which were transferred to minimal medium with 0.05% lactate (w/v) as carbon source in the presence of 5 mM 2- or 3-fluorophenylacetate. Sectors with more vigorous growth were purified and tested for the utilization of different PhAc derivatives as sole carbon source. Two major classes were found, which were denoted class I and II (see “Results”).

**phacA Gene Disruption**—A pUC18-based plasmid denoted pPhAc-argB was constructed by standard techniques. This plasmid contains (starting from the *lacZ* promoter in pUC18) 0.94 kb of the *phacA* upstream region sequentially followed by its genomic coding region up to codon 297, a genomic, 3.2-kbp fragment containing an *argB* allele, the genomic sequence of *phacA* corresponding to codons 393–518, and finally 1.2 kb of the *phacA* 3′-downstream region. This insert was cleaved from the plasmid after digestion with *SmaI* and used for transformation (14). Transformed (arginine-independent) clones in which the resident *phacA* gene had been replaced by the transforming fragment were identified by Southern analysis. The mutated allele would encode a protein truncated at residue 297 and therefore would lack the predicted region involved in heme binding.

**Characterization of *phacA* cDNA and Genomic Clones—** *phacA* cDNA clones were obtained by differential screening of a cDNA library enriched in PhAc-induced transcripts, as described (8–10). Seven *phacA* cDNA clones were obtained. The insert of one such clone was used to isolate genomic clones from a standard *EMBL* library. Cross-hybridizing sequences were mapped to two contiguous *BamHI* fragments, 2.4 kb and 1.9 kb long (a *BamHI* site was shown to split the *phacA* open reading frame). Nucleotide sequencing and comparison of genomic and cDNA clones revealed the intron-exon organization of the gene and showed that all seven *phacA* cDNA clones were incomplete at the N-terminal coding region, the longest of which (at the 5′-end) ended within predicted codon 6. cDNAs including the predicted initiation codon were obtained by direct polymerase chain reaction amplification of the cDNA library using an internal *phacA* primer and *argt*-specific primers following a second polymerase chain reaction reaction using the above internal oligonucleotide and a second oligonucleotide ending 1 nucleotide upstream of the ATG codon. The presence of the ATG codon in the resulting cDNA product (and the absence of introns between codons 1 and 6) was confirmed by sequencing.

**Preparation of Microsomes and Enzyme Assays—** Microsomes from the isogenic *ΔphacA* and *phacA* strains were pregrown in glucose minimal medium, transferred to 10 mM PhAc minimal medium, and incubated for an additional 4 h at 37 °C to induce transcription of genes for PhAc catabolism (13). Cells were collected by filtration, washed, and resuspended in 100 mM potassium phosphate buffer, pH 7.0, and disrupted with a glass bead beater (Braun, 0.5-mm glass beads) at 4 °C. Crude extracts were clarified after centrifugation at 22,000 × g for 15 min. Microsomal pellets were recovered after centrifugation at 100,000 × g for 1 h and resuspended in 100 mM potassium phosphate buffer, pH 7.0. These extracts contained 1–4 mg/ml protein. Enzyme activities of the microsomal extract were determined using standard procedures (15, 16) with minor modifications. NADPH-cytochrome P450 reductase was assayed in 1-ml reactions at 25 °C with 0.1 mM phosphate buffer, pH 7.0, following the NADPH-dependent reduction of cytochrome *c* (0.05 mM initial concentration; *ε*<sub>280</sub> = 21.3 mM<sup>−1</sup> cm<sup>−1</sup>) or ferrocyanide (0.5 mM initial concentration; *ε*<sub>280</sub> = 1.02) by the decrease of absorbance at 550 nm and 420 nm, respectively. PhAc 2-hydroxylase was assayed by measuring the formation of 2-hydroxy-PhAc in a reaction that required PhAc (added at 1 mM) and NADPH (also at 1 mM) in the presence of a microsomal fraction. 2-Hydroxy-PhAc was chemically determined using diazotized 4-nitroaniline procedures (17). Absorbance was read at 550 nm and converted to nmol of 2-hydroxy-PhAc using a reference plot. The range of linear response was 1–100 nmol of 2-hydroxy-PhAc. This method also detected 3-hydroxy-PhAc, and therefore, the identity of the reaction product as 2-hydroxy-PhAc was confirmed by direct HPLC analysis of the reaction mixtures (see “Results”). Proteins were precipitated in the presence of 5% (w/v) trichloroacetic acid, and 10 μl samples were injected into a Nucleosil 300–5 C18 column (250 × 4 mm) coupled to a 11 × 4-mm precursor column of this support, using as mobile phase (at 0.6 ml/min) a solution containing 50 mM monohydrogen potassium phosphate, 0.1 mM EDTA, 100 mM trifluoroacetic acid, and 8% (v/v) acetonitrile. Detection was at 220 nm.

**Penicillin Production—** Cultures of penicillin production were inoculated with spores of the *ΔphacA* or the *phacA*<sup>+</sup> strain in penicillin production broth (18) with 2% lactose as the main carbon source, 2.5% (w/v) corn steep liquor, and the indicated concentrations of sodium phenylacetate. Flasks were shaken at 250 rpm at 37 °C. Samples were taken at different time points and used to measure penicillin using a bioassay with *Micrococcus luteus*, with penicillin G as standard (18).

**RESULTS**

**The “Upper” Phenylacetate Degradation Pathway—** We found that 2-, 3-, or 4-fluoro-PhAc prevents *A. nidulans* growth on 0.05% (w/v) lactose as carbon source. We therefore selected mutations resulting in fluorophenylacetate resistance, assuming that they would prevent PhAc utilization and following Apirion (19), who used fluorocacetate resistance to select acetate nonutilizing mutants. Mutations preventing PhAc utilization (*phac*) were efficiently selected with 2-fluorophenylacetate. They were recessive in diploids, indicating that they represent loss-of-function mutations. They were classified in two major classes. Class I mutants did not grow on PhAc but grew on 2-hydroxy-PhAc or 2,5-dihydroxy-PhAc. By contrast, class II mutants did not grow on either PhAc or 2-hydroxy-PhAc but grew on 2,5-dihydroxy-PhAc. Class I or II mutations did not affect growth on acetate, Phc, Tyr, 3- or 4-hydroxy-PhAc, and 3,4-dihydroxy-PhAc, showing that they specifically prevented PhAc catabolism. Mutations in class I complemented class II mutations. As 2,5-dihydroxy-PhAc is known to be an intermediate of PhAc catabolism (8–10), these data are consistent with the pathway shown in Fig. 1 in which PhAc is converted into homogentisate through two hydroxylating reactions, prevented by class I and class II mutations, respectively.

**Molecular Cloning of *phacA*, a Gene Encoding a Novel Cytochrome P450 in Fungal Phenylacetate Catabolism**

The scheme illustrates the pathway for the degradation of phenylacetate to acetic acid. The upper pathway involves a cytochrome P450 that catalyzes the hydroxylation of PhAc to 2-hydroxy-PhAc. The lower pathway involves a cytochrome P450 that catalyzes the hydroxylation of 2-hydroxy-PhAc to 2,5-dihydroxy-PhAc. The hydroxylation of 2,5-dihydroxy-PhAc to acetic acid is catalyzed by a hydroxysterol 20-hydroxysterol 20-desaturase. The hydroxysterol desaturase is encoded by the *hmgA* gene. The *hmgA* gene is expressed in response to PhAc and is required for the growth of *A. nidulans* on PhAc.

**Fig. 1. The A. nidulans phenylacetate degradation pathway.** Shown are the steps required for PhAc degradation to Krebs cycle intermediates. Enzymes in the upper pathway are specific for PhAc. Enzymes in the lower pathway (gene names italicized) are common to PhAc and Phe/Tyr catabolism (indicated with horizontal arrows). Phe and Tyr are also degraded through homogentisate.

**RESULTS**

The scheme illustrates the pathway for the degradation of phenylacetate to acetic acid. The upper pathway involves a cytochrome P450 that catalyzes the hydroxylation of PhAc to 2-hydroxy-PhAc. The lower pathway involves a cytochrome P450 that catalyzes the hydroxylation of 2-hydroxy-PhAc to 2,5-dihydroxy-PhAc. The hydroxylation of 2,5-dihydroxy-PhAc to acetic acid is catalyzed by a hydroxysterol 20-hydroxysterol 20-desaturase. The hydroxysterol desaturase is encoded by the *hmgA* gene. The *hmgA* gene is expressed in response to PhAc and is required for the growth of *A. nidulans* on PhAc.
DNA fragment carrying a mutated \textit{phacA} argB$^2$ transformed an disruption-deletion volvement of PhacA in PhAc catabolism, we constructed a \textit{phacA} tion to some extent. These results support the contention that \textit{phacA} Encoding a PhAc 2-Hydroxylase—PhAc, 2,5-, or 3,4-dihydroxy-PhAc did not induce PhAc, and 3-hydroxy-PhAc was a weak inducer. 4-Hydroxy- or to gluconeogenic substrates acetate or glutamate. 2-Hy-

FIG. 2. Northern analysis of \textit{phacA} transcript levels. Cells were grown on minimal medium with 0.3\% (w/v) glucose as sole carbon source for 16 h at 37°C and transferred to media with the indicated carbon sources (glucose at 1\% (w/v), all aromatic compounds, and glutamate at 10 mM and potassium acetate at 30 mM; −carbon indicates no carbon source added). These secondary cultures were incubated for a further 1 h at 37°C. Mycelia were then harvested and used to isolate RNA (18). The probe was a 1.2 kb \textit{phacA} cDNA clone (4F4G). Actin transcript was used as loading control.

 sequencing. Seven overlapping cDNAs represented a novel PhAc-induced transcript whose gene was named \textit{phacA} and which contained an open reading frame putatively encoding a 518-residue polypeptide ($M_r$, 58,495). DNA sequencing of genomic clones showed that the \textit{phacA} coding region is interrupted by three introns, 65, 56, and 53 nucleotides long. The nucleotide sequence of \textit{phacA} and the amino acid sequence of its derived protein product have been deposited in the DDBJ/EMBL/GenBank data bases under accession number AJ132442. Blast searches against nonredundant Swissprot+Translation of EMBL nucleotide sequence data bases revealed that all 20 entries showing the highest amino acid sequence identity to PhacA were cytochrome P450 proteins of the CYP1 family (of which 19 were CYP1A1 P450s), including mammalian and fish proteins but only a single human CYP. Identity levels were in the 25\% range, with the highest identity (27.4\% in a 465 residue overlap) shown by a \textit{Sparus aurata} (gilthead sea bream) P450 protein. Cytochromes P450 are heme-thiolate en-

FIG. 3. Disruption of \textit{phacA}. The \textit{phacA} gene was replaced by a mutant version after transformation with a linear DNA fragment in which \textit{phacA} codons 298−392 had been replaced by a 3.2-kbp DNA fragment containing the \textit{argB} gene (see “Experimental Procedures”). This mutant allele encodes a PhacA protein truncated at residue 297. Two transformants (denoted \textit{\Delta phacA} #3 and #4) carrying the expected disruption-deletion mutation were purified and tested for growth on minimal medium with 0.05\% (w/v) lactate, 10 mM PhAc, or 10 mM 2-hydroxy-PhAc as sole carbon source, as indicated. A wild type strain and a strain carrying a null (\textit{\Delta mngA}) mutation in the homogenisate dioxygenase gene (see Fig. 1) were used as controls. Plates were incubated for 4 days at 37°C before being photographed.

3.2-kbp fragment containing an \textit{argB} allele replaced a 289-base pair \textit{NaeI-Kpn1} \textit{phacA} genomic fragment including codons 298−392 (see Fig. 3). This mutant \textit{phacA} gene would encode a PhacA protein truncated at residue 297 and therefore lacking the 221 C-terminal residues, which include the essential Cys-containing peptide motif involved in heme binding. Homokary-

otic transformant clones were purified after repeated streaking on medium lacking arginine and analyzed by Southern blot hybridization. Two transformants showing an identical hybridiz-

ation pattern consistent with the integration event shown in Fig. 3 were chosen for further analyses. Both grew normally on the 0.5\% (w/v) lactate but, in contrast to the wild type, showed residual growth (similar to that observed in the absence of a carbon source) on PhAc-minimal medium (Fig. 3), showing that PhacA is indeed involved in PhAc catabolism. By contrast, both transformants were able to grow on 2-hydroxy-PhAc (Fig. 3). These data indicate that PhacA is involved in the \textit{ortho}-hydroylation of PhAc. The disruption-deletion mutation (denoted \textit{\Delta phacA}) was recessive in diploids (in agreement with its predicted loss-of-function phenotype) and did not complement \textit{phac}-4, a prototype of classical class I mutations that, in common with \textit{\Delta phacA}, leads to inability to use PhAc but allows growth on 2-hydroxy-PhAc. \textit{phac}-4 is therefore a \textit{phacA} allele that we renamed \textit{phacA}.

\textit{\Delta phacA} Microsomes Are Deficient in P450 PhAc 2-Hydroxylating Activity—Most eukaryotic cytochromes P450 are micro-

somal enzymes. Electrons are transferred to their catalytic heme center from (NADPH)-cytochrome P450 reductase, a micro-

somal enzyme containing FAD and FMN (see Fig. 4A). We prepared microsomal fractions from wild type and \textit{phacA} cells induced with PhAc, which showed similar (NADPH)-cyto-

chrome P450 reductase activity, as assayed by the NADPH-de-

pendent reduction of artificial electron acceptors such as ferricyanide or cytochrome c (20) (Fig. 4A). The corresponding \textit{phacA} and \textit{\Delta phacA} soluble fractions showed similar, high levels of homogentisate dioxygenase (a soluble enzyme of the PhAc degradation pathway, data not shown), indicating the equivalent induction of PhAc catabolism in both strains.

Following incubation in vitro of wild type microsomes with

\textsuperscript{2} David R. Nelson, personal communication.
PhAc and NADPH, we detected the formation of a monohydroxylated PhAc derivative using a chemical detection method (Fig. 4A; see “Experimental Procedures”). Direct HPLC analysis of the reaction mixture showed that this compound was 2-hydroxy-PhAc and that its formation was absolutely dependent on the presence of both PhAc and NADPH (Fig. 4B). This showed that \textit{A. nidulans} microsomes contain a phenylacetate ortho-hydroxylating activity. This activity was markedly and reproducibly reduced but not abolished by the \textit{DphacA} mutation (Fig. 4, A and B). Finally, wild type mycelia pregrown in glucose and transferred to media containing PhAc secreted 2-hydroxy-PhAc to the culture supernatant (Fig. 5). In agreement with the above \textit{in vitro} assays, secretion of 2-hydroxy-PhAc was also markedly reduced, but not abolished, by the \textit{DphacA} mutation (Fig. 5). All these data, together with the growth characteristics of the \textit{DphacA} strain (see above), strongly support the conclusion that \textit{phacA} encodes a phenylacetate 2-hydroxylase and that a second, minor enzyme showing this activity is present in \textit{A. nidulans} microsomes (see “Discussion”).

\textbf{FIG. 4.} \textit{phacA} disruption results in a marked reduction of microsomal PhAc 2-hydroxylase activity. A, a cytochrome P450 monooxygenase catalyzes the incorporation of one of the atoms from dioxygen (as an hydroxyl group) into the aromatic ring of PhAc. The source of electrons for such reaction is a NADPH-cytochrome P450 oxidoreductase, an enzyme that transfers electrons from NADPH through two flavin redox centers (20). \textit{In vitro}, the activity of the reductase can be monitored by using artificial electron acceptors such as ferricyanide and cytochrome c, as indicated. Shown below are PhAc 2-hydroxylase and NADPH-cytochrome P450 oxidoreductase activities in the microsomal fractions of \textit{DphacA} and \textit{phacA} strains. Formation of 2-hydroxy-PhAc was monitored with a chemical method (17). B, HPLC analysis of PhAc 2-hydroxylase in the above microsomal fractions. The positions of standards were indicated by roman numbers as follows: I, 2,5-dihydroxy-PhAc; II, 3,4-dihydroxy-PhAc; III, 4-hydroxy-PhAc; IV, 3-hydroxy-PhAc; V, 2-hydroxy-PhAc; VI, PhAc. The retention time for authentic 2-hydroxy-PhAc was 11.82 min, whereas the product formed in the complete \textit{phacA} and \textit{DphacA} reactions showed retention times of 11.81 and 11.86 min, respectively.

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\textbf{FIG. 5.} Reduced secretion of 2-hydroxyphenylacetate in a \textit{DphacA} strain. Culture supernatants of \textit{phacA} (circles) and \textit{DphacA} (triangles) strains were assayed for the presence of 2-hydroxy-PhAc. Mycelia were pregrown in glucose minimal medium and transferred to PhAc. Samples were taken at the indicated time-points after the transfer.
experiments, illustrating how the increase is already evident in cultures supplemented with 0.125% (w/v) PhAc (1.8 µg/ml in the wild type, 5.2 µg/ml in the ΔphacA strain). Moreover, reduction of PhAc to 0.0625% (w/v) resulted in nearly a 40% decrease in wild type penicillin production but had no decrease in the ΔphacA strain. Under such conditions, the ΔphacA mutation abolished microsomal PhAc 2-hydroxylation. A ΔphacA strain grows on PhAc but does not grow on 3-hydroxy-PhAc (which in the wild type is also catabolized through homogenisate), indicating that phacA encodes a 3-hydroxy-PhAc 6-hydroxylase (i.e. and ortho-hydroxylase), converting 3-hydroxy-PhAc to 2,5-dihydroxy-PhAc. This enzyme has been previously described in the fungus Trichosporon cutaneum, which converts PhAc to homogenisate through sequential hydroxylation of positions 3 and 6 of the ring (22).

Higher eukaryotes have multiple cytochrome P450 monoxygenases catalyzing a variety of oxidative reactions. Such abundance is not found in the microbial world. For example, only three CYP genes are found in the genome of S. cerevisiae (23). The marked metabolic versatility of filamentous fungi would suggest a greater variety of CYP enzymes in their proteomes. In addition to phacA, four ste genes of the A. nidulans sterigmatocystin biosynthetic cluster encode CYP enzymes (24). The closely related organism A. niger has a very specific benzoate-4 hydroxylase enzyme, encoded by the bpha gene (25). Notably, PhAc and Bpha each define a new CYP family, suggesting that metabolically versatile saprophytic fungi may represent an as yet unexplored source of variability for CYP enzymes catalyzing novel metabolic reactions.

Industrial penicillin production by Penicillium chrysogenum strains requires the addition of PhAc, which is the side-chain precursor for the synthesis of penicillin G. Part of the added PhAc is oxidized (26), and it is not transformed into penicillin. Therefore, strain improvement programs using mutation and selection techniques have been directed to prevent such oxidation (27). Engineered expression in P. chrysogenum of a bacterial phenylacetyl-CoA ligase leading to increased levels of phenylacetyl-CoA available for penicillin biosynthesis resulted in penicillin overproduction (28). We show here that targeted disruption of the gene mediating the first step of A. nidulans PhAc catabolism results in a 3- to 5-fold increase in penicillin production and makes the recombinant strain less dependent on the external supply of PhAc. This presumably results from increased availability of phenylacetate for penicillin G biosynthesis. A P. chrysogenum phacA homologue has been identified. Therefore, these results pave the way for the improvement of P. chrysogenum industrial strains using a similar methodology.

Acknowledgments—We thank E. Reoyo for technical assistance, Brian Nowak-Thompson, Eduardo Díez, and José Luis García for critical reading of the manuscript, Beatriz Galañ and Auxi Prieto for their advice with HPLC analysis, and David R. Nelson for his assignment of an standarized cytochrome P450 designation to PhacA.

REFERENCES
1. Harayama, S., Kok, M., and Neidle, E. L. (1992) Annu. Rev. Microbiol. 46, 565–601
2. Harayama S., and Timmis K. N. (1992) in Metal Ions in Biological Systems (Sigel H., and Sigel A., eds) pp. 99–155, Marcel Dekker Inc., New York
3. Schreuder, H. A., Prick, P. A., Wierenga, R. K., Vriend, G., Wilson, K. S., Hol, W. G., and Drenth, J. (1989) J. Mol. Biol. 208, 679–696
4. Whited, G. M., and Gibson, D. T. (1991) J. Biological Chem. 266, 3010–3016
5. Nelson, D. R., Koymans, L., Kamataki, T., Stegeman, J. J., Feyerisen, R., Waxman, D. J., Waterman, M. R., Gotsh, O., Coon, M. J., Estabrook, R. W., Gunsalus, I. C., and Nebert, D. W. (1996) Pharmacogenetics 6, 1–42
6. Nebert, D. W., and Gonzalez, F. J. (1987) Annu. Rev. Biochem. 56, 945–993
7. Olivera, E. R., Minambres, B., Garcia, B., Muniz, C., Moreno, M. A., Fernandez-Cano, J. D., Garcia, J. L., and Luengo, J. M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9132–9136
8. Fernandez-Cano, J. M., and Peñalva, M. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9132–9136
9. Fernandez-Cano, J. M., and Peñalva, M. A. (1995) J. Biol. Chem. 270, 21199–21205
10. Fernandez-Cano, J. M., and Peñalva, M. A. (1998) J. Biol. Chem. 273, 329–337
11. Mingot, J. M., Peñalva, M. A., and Fernandez-Cano, J. M. (1998) unpublished results.
11. Clutterbuck, A. J. (1993) in *Genetic Maps. Locus Maps of Complex Genomes* (O’Brien, S. J., ed) pp. 3.71–3.84, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
12. Cove, D. J. (1966) *Biochim. Biophys. Acta* **113**, 51–56.
13. Fernández-Cañón, J. M., and Peñalva, M. A. (1997) *Anal. Biochem.* **245**, 218–221.
14. Tilburn, J., Scanzacchio, C., Taylor, G. G., Zabicky-Zissman, J. H., Lockington, R. A., and Davies, R. W. (1983) *Gene* **26**, 205–211.
15. Strobel, H. W., and Dignam, J. D. (1978) *Methods Enzymol.* **52**, 89–96.
16. Benveniste, I., Lesot, A., Hasenfratz, M. P., and Duret, P. (1989) *Biochim. J.* **259**, 847–853.
17. Sugumaran, M., and Vaidyanathan, C. S. (1979) *FEMS Microbiol. Lett.* **5**, 427–430.
18. Espeso, E. A., and Peñalva, M. A. (1992) *Mol. Microbiol.* **6**, 1457–1465.
19. Apirion, D. (1965) *Genet. Res.* **6**, 317–329.
20. Vermilion, J. L., and Conon, M. J. (1978) *J. Biol. Chem.* **253**, 8812–8819.
21. Luengo, J. M., and Peñalva, M. A. (1994) in *Aspergillus: 50 Years On* (Martinelli, S. D., and Kinghorn, J. R., eds) pp. 603–638, Elsevier Science Publishers B.V., Amsterdam.
22. Anderson, J. J., and Dagley, S. (1980) *J. Bacteriol.* **141**, 534–543.
23. van den Brink, H. M., van Gorcom, R. F., van den Hondel, C. A., and Punt, P. J. (1998) *Fungal Genet. Biol.* **25**, 1–17.
24. Brown, D. W., Yu, J. H., Kelkar, H. S., Fernandes, M., Neshitt, T. C., Keller, N. P., Adams, T. H., and Leonard, T. J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 1418–1422.
25. van Gorcom, R. F., Boschloo, J. G., Kuijvenhoven, A., Lange, J., van Vark, A. J., Bos, C. J., van Balken, J. A., Pouwels, P. H., and van den Hondel, C. A. (1990) *Mol. Gen. Genet.* **223**, 192–197.
26. Hockenhull, D. J. D., Walker, A. D., Wilkin, J. D., and Winder, F. G. (1951) *Fungal Genet. Biol.* **23**, 1–17.
27. Lein, J. (1986) in *Overproduction of Microbial Metabolites* (Vanek, Z., and Hostalek, Z., eds) pp. 105–139, Butterworths, Stoneham, MA.
28. Minambres, B., Martinez Blanco, H., Olivera, E. R., Garcia, B., Diez, B., Barredo, J. L., Moreno, M. A., Schleissner, C., Salto, F., and Luengo, J. M. (1996) *J. Biol. Chem.* **271**, 33531–33538.