Distinct Roles for Two CYP226 Family Cytochromes P450 in Abietane Diterpenoid Catabolism by *Burkholderia xenovorans* LB400[∇]

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The 80-kb *dit* cluster of *Burkholderia xenovorans* LB400 encodes the catabolism of abietane diterpenoids. This cluster includes *ditQ* and *ditU*, predicted to encode cytochromes P450 (P450s) belonging to the poorly characterized CYP226A subfamily. Using proteomics, we identified 16 *dit*-encoded proteins that were significantly more abundant in LB400 cells grown on dehydroabietic acid (DhA) or abietic acid (AbA) than in succinate-grown cells. A key difference in the catabolism of DhA and AbA lies in the differential expression of the P450s; *DitU* was detected only in the AbA-grown cells, whereas *DitQ* was expressed both during growth on DhA and during growth on AbA. Analyses of insertion mutants showed that *ditQ* was required for growth on DhA, *ditU* was required for growth on AbA, and neither gene was required for growth on the central intermediate, 7-oxo-DhA. In cell suspension assays, patterns of substrate removal and metabolite accumulation confirmed the role of *DitU* in AbA transformation and the role of *DitQ* in DhA transformation. Spectral assays revealed that *DitQ* binds both DhA (dissociation constant, $0.98 \pm 0.01 \mu\text{M}$) and palustric acid. Finally, *DitQ* transformed DhA to 7-hydroxy-DhA in vitro. These results demonstrate the distinct roles of the P450s *DitQ* and *DitU* in the transformation of DhA and AbA, respectively, to 7-oxo-DhA in a convergent degradation pathway.

The terpenoids are the largest family of natural products, which includes more than 25,000 individual compounds. These compounds are synthesized by most, if not all, organisms, but they are most notably made by plants and function as hormones and defense compounds (19, 40). Because of their diverse biological activities, many terpenoids are employed as chemotherapeutic drugs (29). Unfortunately, the natural sources of such drugs are often inadequate due to the limited quantities produced and inefficient purification methods, as illustrated by taxol, which cannot be sustainably produced by the Pacific yew in the quantity demanded (20). An improved understanding of the biosynthesis and enzymatic transformations of terpenoids would facilitate efficient production of large quantities of useful compounds, such as amorphadiene (24) and taxol (8).

Cytochromes P450 (P450s) are involved in both biosynthesis and biodegradation of terpenoids. All terpenoids are synthesized from five-carbon precursors via a limited range of modifications (11, 33). Particular terpenoids require further modifications involving cyclization and other changes to the C skeleton (21). P450-catalyzed monooxidations are commonly employed to effect stereospecific and regiospecific addition of O-containing functional groups or to otherwise modify the C skeleton (14, 18, 32). Microorganisms and animals employ

P450s to transform terpenoids, mainly to reduce the compounds' toxicity, to increase their solubility, or to exploit them as growth substrates.

There is limited evidence for the involvement of P450s in the microbial catabolism of resin acids, a class of diterpenoids synthesized mainly by trees for defense against insects and microorganisms. Because of their abundance in wood and their toxicity to fish, resin acids are important pollutants resulting from pulp and paper production (1). A wide range of bacteria are known to grow on abietane resin acids (26, 42), and recent studies have provided evidence for the involvement of P450s in bacterial catabolism of these compounds. Knockout mutagenesis in *Pseudomonas diterpeniphila* A19-6a indicated that the P450 TdtD (CYP226A3) is involved in, but is not essential for, catabolism of dehydroabietic acid (DhA) and abietic acid (AbA) (27). Knockout mutagenesis of *Pseudomonas abietaniphila* BKME-9 revealed that a TdtD homolog, *DitQ*, is involved in, but is not essential for, catabolism of DhA and palustric acid (PaA) but is not involved in catabolism of AbA (39). Metabolites from the BKME-9 *ditQ* disruption mutant and a binding assay with heterologously expressed *DitQ* suggested that this enzyme catalyzes hydroxylation of C-7 of DhA, but there is no direct experimental evidence for this reaction.

Burkholderia xenovorans LB400 is a member of a widespread group of soil bacteria frequently associated with plants. Recent investigation of LB400 identified a large cluster of abietane catabolic genes and suggested that bacteria may employ multiple P450s to degrade the diterpenoids (38). The LB400 gene cluster encodes two homologs of *DitQ*_{BKME-9}: *DitQ*_{LB400} (CYP226A1) and *DitU* (CYP226A2). All three P450s share greater than 60% amino acid identity. This is a very high level of identity for this class of enzymes, as homologous P450s commonly share only 40% identity. The possible presence of

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
<i>Burkholderia xenovorans</i> strains		
LB400	Wild type; grows on abietane diterpenoids	16
DitQKO	<i>ditQ::xylE-accC1</i> ; Gm ^r	This study
DitUKO	<i>ditU::xylE-accC1</i> ; Gm ^r	This study
<i>Escherichia coli</i> strains		
DH5 α	<i>endA1 hsdR17(r_K⁻ m_K⁻) supE44 thi-1 recA1 gyrA (Nal^r) relA1 Δ(<i>lacIZYA-argF</i>)U169</i>	Gibco BRL
S17-1	<i>deoR</i> [ϕ 80 <i>dlac</i> Δ (<i>lacZ</i>)M15] <i>recA pro thi hsdR</i> with integrated RP4-2-TcMu::Kna::Tn7; Tra ⁺ Tp ^r Sm ^r	37
Plasmids		
pEX18AP	<i>sacB</i> conjugatable plasmid for gene replacement; Ap ^r	34
pX1918G	<i>xylE</i> -Gm ^r fusion cassette-containing plasmid; Ap ^r Gm ^r	34
pEX5906	1.3-kbp PCR amplicon containing LB400 <i>ditQ</i> cloned into the XbaI-HindIII site of the multiple-cloning site of pEX18AP	This study
pEX5906KO	PCR-amplified <i>xylE</i> -Gm ^r fusion cassette of pX1918G cloned into the KasI-NcoI site of pEX5906	This study
pEX5938	1.6-kbp PCR amplicon containing LB400 <i>ditU</i> cloned into the EcoRI-BamHI site of the multiple-cloning site of pEX18AP	This study
pEX5938KO	XmaI-digested fragment of pX1918G containing the <i>xylE-accC1</i> fusion cassette cloned into the unique XmaI site of pEX5938, disrupting <i>ditU</i>	This study

functional P450 homologs with complementary activities may explain how the P450 knockout strains described above retained a capacity for reduced growth on abietanes. However, transcriptomic analysis of LB400 indicated that *ditQ*, but not *ditU*, was up-regulated during growth of LB400 on DhA (38). Thus, the patterns of expression and substrate specificities of various P450s likely dictate distinct roles for the enzymes in abietane metabolism, and these roles remain to be elucidated. All of the P450s implicated in abietane metabolism represent a distinct subfamily, the CYP226A subfamily. This subfamily is in the broader CYP226 family, for which the only published information is from the studies of TdtD (27) and DitQ_{BKME-9} (39) mentioned above.

Here we describe the first proteomic investigation of microbial catabolism of abietane diterpenoids, which was aimed at clarifying the roles of P450s in the initial catabolic steps. Two-dimensional gel-based proteomic analysis of LB400 grown on DhA or on AbA revealed differential expression of the P450s DitQ and DitU. Further analysis involved characterization of LB400 *ditQ* and *ditU* disruption mutants, as well as expression of each P450 in *Escherichia coli*, followed by substrate-binding and activity assays of the expressed enzymes. This study provides the first conclusive evidence that two P450s have distinct

functions in abietane diterpenoid catabolism and the first demonstration of a catalytic activity of a CYP226 family member.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains used in this study are listed in Table 1. *E. coli* was cultured on Luria-Bertani medium and incubated at 37°C. *B. xenovorans* strains were cultured at 30°C on Luria-Bertani medium without NaCl or on K1 mineral medium (9) supplemented with 1 g succinate per liter, 90 mg AbA per liter, 90 mg DhA per liter, 90 mg PaA per liter, or 95 mg 7-oxo-DhA per liter. All liquid cultures were incubated on a rotary shaker at 200 to 250 rpm.

The cultures of LB400 or mutants DitQKO and DitUKO were initially grown on solid K1 medium with biphenyl as the sole organic substrate. Individual colonies were then inoculated into 50 ml of K1 medium with succinate as an organic substrate. Late-log-phase (optical density at 600 nm, 0.9) cells were sequentially transferred two times to fresh succinate medium. A third transfer was then grown in 1 liter of succinate medium to mid-log phase (optical density at 600 nm, 0.5 to 0.6) and harvested. Cells from the second sequential succinate cultures were also used to inoculate 200 ml of K1 medium containing AbA, DhA, PaA, or 7-oxo DhA. These cultures were sequentially transferred on their respective growth substrates three times (with the third transfer into 1 to 3 liters of medium), grown to mid-log phase, and harvested. Growth characteristics were determined for each substrate on which growth occurred, using cultures grown for the third time on that substrate. All experiments were done in triplicate using independent cultures.

TABLE 2. Oligonucleotide primers used in PCR

Primer	Sequence ^a	Restriction site
HISLEFT5906	5'-CGC GCG CTA GCA TGG AGA CCG GAA TGA CCA-3'	NheI
HISRIGHT5906	5'-GCG CGA AGC TTG TCG ACG GAC TAC CGC TCA-3'	HindIII
XyLE5906L	5'-GAT CCC ATG GAC CGT GAT CGG CGA ACT GGA-3'	NcoI
XyLE5906R	5'-ATA TGG CGC CCTCGG CCA CCG TCA TCT TCC-3'	KasI
KOLBcep5938	5'-CGC GGA TCC TGG TGA GTT GCA GGC CGT A-3'	BamHI
KORBcep5938	5'-CCG GAA TTC ACG CCG TTA AGC TGC ACG A-3'	EcoRI
HISLEFT5938	5'-CGC GCA AGC TTT GGT GAG TTG CAG GCC GTA-3'	HindIII
HISRIGHT5938	5'-CGC GCG CTA GCA TGA GCA CCA CCC TCG AAA-3'	NheI

^a Restriction sites are underlined.

Gene replacement. The gene encoding DltQ and the gene encoding DltU were disrupted by replacement as previously described (38). The primers and plasmids used are listed in Tables 1 and 2. The knockouts were confirmed by PCR using primers XylE5906R and HISLEFT5906 (for DltQKO) and HISLEFT5938 and HISRIGHT5938 (for DltUKO). Amplicons generated from both DltQKO and DltUKO were sequenced and were found to contain the expected product.

Cell suspension assays. Cell suspension assays were conducted as previously described (38). Cells were cultured on succinate as described above. Cell suspensions were supplemented with 300 μ M AbA, DhA, PaA, or 7-oxo-DhA. Controls contained cells killed by boiling. Samples were derivatized using diazo-methane and were analyzed by gas chromatography (GC)-mass spectrometry (MS). GC electron impact MS of the derivatives was conducted as previously described (39), using an Agilent Technologies 6890N network GC system equipped with an Agilent 5973 mass selective detector. National Institute of Standards and Technology MS search (version 2.0) was used to analyze mass spectral data. All experiments were performed in triplicate with cells from independent cultures. Replicate experiments yielded the same patterns of substrate removal and metabolite accumulation.

Proteomic analyses. Cell extracts were prepared, and the cytosolic proteome was resolved using two-dimensional gel electrophoresis essentially as described previously (10, 30). Proteins were separated in the first dimension by isoelectric focusing using 24-cm, nonlinear IPG strips with a pH 3 to 7 gradient and in the second dimension using 12% sodium dodecyl sulfate-polyacrylamide gels and the ETTAN DALT twelve system (GE Healthcare). Gels were stained using Sypro Ruby and were digitally imaged using a Typhoon 9400 (GE Healthcare). Protein spot detection and pattern matching were performed using Progenesis workstation software (Nonlinear Dynamics, Durham, NC), and then the results were verified manually. The patterns for each growth condition were based on gels from three independent cultures. The signal intensity of each spot was normalized using the total signal intensity detected on a gel. Only spots with a minimum normalized volume of 0.002 or more were analyzed further. Experimental molecular mass and isoelectric point values were assigned using the Progenesis calibration algorithm. Theoretical molecular mass and isoelectric point values were predicted based on protein sequences using the ExPASy pI/M_w tool, available at http://ca.expasy.org/tools/pi_tool.html. Protein spots of interest were identified using a Voyager DESTR matrix-assisted laser desorption/ionization-time of flight mass spectrometer (Applied Biosystems, Foster City, CA) based on peptide mass fingerprint analysis combined with the MASCOT search engine (www.matrixscience.com) and the LB400 protein database generated at the Proteomics Centre, University of Victoria. The proteins identified fulfilled four criteria: the MASCOT search score was greater than 52, a minimum of six peptides were matched, the protein sequence coverage was at least 20%, and the predicted mass and pI values were consistent with the experimentally determined values.

Substrate binding. Cultures of *E. coli* DH5 α containing pEX5938, pEX5906, or pEX18Ap were prepared and analyzed as described previously (39), with the following exception. Instead of a French press, two passes through an Emulsiflex C-5 cell disrupter operated at a homogenizing pressure of 15,000 lb/in² were used for cell lysis. DltQ was assayed at concentrations of 0.46 to 0.77 μ M, and DltU was assayed at concentrations of 0.61 to 0.70 μ M.

In vitro P450 assay. Crude lysates of *E. coli* expressing DltQ or DltU were collected and quantified as described previously for substrate binding assays (39). Ferredoxin DltA3 from *P. abietaniphila* BKME-9 and ferredoxin reductase BphG from *Comamonas testosteroni* were each purified to homogeneity, as previously described (6, 15). Vials containing both DltA3 and BphG were sparged with argon for approximately 1 min prior to the activity assay. BphG (1.8 μ M), DltA3 (3.6 μ M), crude lysate of *E. coli* expressing DltQ or DltU (1.0 μ M), NADH (350 μ M), and 100 μ M DhA, AbA, or PaA were combined and incubated for 5 min at room temperature or for 30 min at 30°C. The controls included an acidified reaction mixture or crude lysate from isopropyl- β -D-thiogalactopyranoside (IPTG)-induced *E. coli* containing pEX18Ap.

RESULTS

AbA and DhA proteomes. Approximately 800 protein spots were detected in the cytosolic proteomes of LB400 grown on succinate, AbA, or DhA as a sole organic substrate. Pairwise comparisons revealed that the greatest similarity was between the DhA and AbA proteomes, in which 84% of the protein spots were shared (Fig. 1). In contrast, the DhA and AbA proteomes shared 75 and 78% proteins, respectively, with the

FIG. 1. Global analysis of LB400 cytosolic proteomes: Venn diagram representation of the protein spots whose normalized intensity was greater than 0.002. The numbers in brackets indicate the total numbers of protein spots detected in the corresponding average gels using Progenesis software.

succinate proteome. Quantitative comparison of spot intensities indicated that of the 679 protein spots common to the DhA and AbA proteomes, 128 proteins were not detected in the succinate proteome and an additional 52 proteins were at least twofold more abundant during growth on at least one abietane diterpenoid than during growth on succinate. Despite the high similarity of the cytosolic proteomes of LB400 grown on DhA and AbA, ~10% of each proteome was unique to DhA or AbA, indicating that these compounds had distinct physiological effects.

Identification of proteins. Among the 180 protein spots that were at least twofold more abundant during growth on at least one diterpenoid substrate, we identified 23 proteins that were most differentially expressed and abundant (Table 3). Sixteen of the identified proteins were encoded in the *dlt* gene cluster (BxC0578 to BxC0649), which was previously shown to encode catabolism of abietane diterpenoids (38). These proteins include two P450s. The first P450, DltQ, encoded by BxC0599, was detected in both the AbA and DhA proteomes. The second, DltU, encoded by BxC0631, was unique to the AbA proteome. A minor spot in the DhA and succinate proteomes occurred near the location of DltU, but we excised the protein from both gels and identified it as citrate synthase I. The tryptic digest of this minor spot contained no peptide fragments corresponding to DltU.

With one exception, all other detected proteins encoded by the *dlt* gene cluster were in both DhA and AbA proteomes but not in the succinate proteome. The exception was a conserved hypothetical protein, encoded by BxC0630, adjacent to *dltU*. Like DltU, the product of BxC0630 was detected only in the AbA proteome and not in the DhA or succinate proteome. The identified proteins not encoded in the *dlt* gene cluster included six catabolic enzymes and a transcriptional regulator (Table 3). These seven proteins were similarly abundant in the DhA and AbA proteomes, and most were not detected in succinate-grown cells.

Growth of mutant strains. To investigate the functions of the identified P450s in abietane diterpenoid catabolism, two mutant strains were generated, DltQKO and DltUKO, in which *dltQ* and *dltU*, respectively, were disrupted. The mutants had distinct growth phenotypes. The disruption of *dltQ* com-

TABLE 3. Proteins involved in abietane diterpenoid catabolism identified by MASCOT-based analysis of matrix-assisted laser desorption ionization–time of flight spectra

Gene no. ^a	Gene	Protein	Identification ^b		Normalized signal intensity ^c		
			No. of peptides matched	Sequence coverage	Succinate	DhA	AbA
BxeC0585	<i>ditH</i>	Putative hydrolase	16	57	ND	0.47	0.57
BxeC0586	<i>ditA1</i>	Alpha subunit of ring-hydroxylating dioxygenase	9	26	ND	1.00	1.26
BxeC0587	<i>ditA2</i>	Beta subunit of ring-hydroxylating dioxygenase	8	61	ND	0.89	0.72
BxeC0592	<i>ditJ</i>	Putative coenzyme A ligase	9	20	ND	0.57	0.19
BxeC0594		Conserved hypothetical protein	9	32	ND	1.06	1.24
BxeC0597	<i>ditO</i>	Putative thiolase	11	35	ND	0.32	0.46
BxeC0599	<i>ditQ</i>	Cytochrome P450	17	42	ND	0.15	0.34
BxeC0602		Putative acyl coenzyme A dehydrogenase	12	38	ND	0.68	0.35
BxeC0606		Conserved hypothetical protein	10	33	ND	2.02	2.17
BxeC0619		Putative short-chain dehydrogenase	8	43	ND	0.19	0.17
BxeC0630		Conserved hypothetical protein	7	23	ND	ND	0.42
BxeC0631	<i>ditU</i>	Cytochrome 450	23	64	ND	ND	0.50
BxeC0639	<i>ditB</i>	Putative dehydrogenase	11	66	ND	0.40	0.86
BxeC0640	<i>ditC</i>	Ring cleavage dioxygenase	13	35	ND	0.69	0.46
BxeC0644		Conserved hypothetical protein	13	51	ND	1.41	LR
BxeC0647		Conserved hypothetical protein	17	49	ND	0.42	0.24
BxeB0962		Putative coenzyme A hydratase	14	70	ND	0.16	0.19
BxeB0963		Putative biotin carboxylase	19	36	ND	0.16	0.13
BxeC1186	<i>bphD</i>	Hydrolase	6	25	0.01	0.12	0.26
BxeB1203		Putative isocitrate lyase and phosphorilmutase family protein	10	56	0.04	0.55	0.43
BxeA1366		Putative 3-oxoacid coenzyme A-transferase alpha subunit	7	67	0.05	0.43	0.27
BxeB2301		Aconitate hydratase	32	47	ND	0.56	0.50
BxeA2466		Putative LysR-type regulator	11	38	ND	0.42	LR

^a The LB400 genome sequence is available at <http://genome.ornl.gov/microbial/bfun/>.

^b Number of peptides matched and sequence coverage in MASCOT analysis.

^c Average normalized signal intensities for three biological replicates under each of the growth conditions tested. ND, not detected; LR, low resolution in the area where a spot was expected.

pletely abolished the ability of LB400 to grow on DhA, indicating the essential role of this gene in DhA catabolism (Fig. 2). This mutation also increased the lag phase prior to growth on AbA and PaA by approximately 100 h and increased the doubling time on PaA 2.0-fold. By contrast, the disruption of *ditU* abolished the ability of LB400 to grow on AbA, indicating the essential role of this gene in AbA catabolism. This mutation also increased the lag phase prior to growth on PaA by approximately 100 h and increased the doubling time on PaA 1.5-fold. Growth on the catabolic intermediate, 7-oxo-DhA, was not affected by disruption of either *ditQ* or *ditU*, and neither mutation had a major effect on the growth yield with any substrate, except when growth was completely abolished.

Cell suspension assays. To further investigate the catabolic roles of the two P450s in the catabolism of abietane diterpenoids, we examined substrate removal and metabolite production by whole cells. As previously observed with other strains (25), some initial removal (<20%) of diterpenoids by boiled control cells was detected, likely due to sorption of the diterpenoids to biomass. However, this removal did not continue throughout the incubations and was clearly distinguishable from removal due to biotransformation. Importantly, metabolite production was not observed in boiled controls, indicating that metabolites did not result from abiological processes.

The disruption of *ditQ* did not affect the ability of LB400 to degrade AbA (Fig. 3); however, DitUKO cells removed only 34% of AbA within 72 h. This further confirmed that *ditU*, but

not *ditQ*, is required for the catabolism of AbA. Since 7-oxo-DhA is a minor contaminant of the AbA reagent, trace amounts of 7-oxo-DhA were detected in all samples with AbA. The LB400 and DitQKO cells incubated with AbA accumulated additional 7-oxo-DhA, whereas with DitUKO the initial concentration of this compound did not change. This suggests that 7-oxo-DhA is a metabolite that is normally formed during AbA catabolism by a process requiring *ditU*. Additionally, small amounts of 7-oxo-PaA accumulated in incubations of all three strains with AbA. Both these metabolites were previously observed during diterpenoid catabolism and were identified on the basis of their mass spectra (38).

By contrast, disruption of *ditU* did not markedly affect the ability of LB400 to degrade DhA, but disruption of *ditQ* did affect this ability (Fig. 3). Whereas DitUKO completely removed DhA within 72 h, DitQKO removed only 40% of the DhA in this time. While degrading DhA, both LB400 and DitUKO accumulated small amounts of 7-oxo-DhA; however, DitQKO did not accumulate any detectable metabolites from DhA.

During 72 h of incubation with PaA, LB400 and DitQKO removed most of the PaA, whereas DitUKO removed only a minor fraction (Fig. 3). With a longer incubation time (144 h) (not shown), LB400 completely removed the PaA, whereas DitQKO and DitUKO did not. Since DhA is a contaminant of the PaA reagent, small amounts of DhA were initially present in all treatments with PaA. All strains accumulated additional DhA as well as 7-oxo-DhA and 7-oxo-PaA. All the strains

FIG. 2. Growth characteristics of LB400 (WT) and two mutant strains, DitUKO (DitU) and DitQKO (DitQ), on four abietane diterpenoids. The initial inoculum for each culture was $\sim 2 \times 10^6$ cells/ml grown to mid-log phase on succinate. N indicates no growth. The error bars indicate standard errors.

tested completely removed 7-oxo-DhA within 24 h (data not shown), clearly indicating that neither DitQ nor DitU is essential for catabolism of 7-oxo-DhA.

Binding assays. To confirm that *ditQ* and *ditU* encode functional P450s, carbon monoxide binding assays were conducted. Both DitQ and DitU, produced in a crude lysate of *E. coli*, bound carbon monoxide and yielded a Soret maximum absorbance between 447 and 450 nm with little absorbance at 420 nm (not shown). This indicates that both DitQ and DitU are members of the P450 superfamily and are expressed in *E. coli* in a native conformation.

To investigate the range of compounds that bind to DitQ and DitU, substrate-binding assays were conducted. Addition of DhA to a solution of DitQ induced a type I difference spectrum typical of substrate binding with a maximum at 388 nm and a minimum at 421 nm (Fig. 4A). Titration with DhA yielded a dissociation constant (K_d) of $0.98 \pm 0.01 \mu\text{M}$ (Tris-HCl buffer, pH 7.4). PaA also induced a type I-like curve for DitQ, indicating that PaA is also a substrate for this P450 (Fig. 4B). However, an isosbestic point at 407 nm was not observed, and therefore the K_d could not be determined. AbA induced changes in the spectrum of DitQ, suggesting that it bound to the enzyme, perturbing the heme environment. However, the binding equation could not be fitted to the data, and therefore

the results were inconclusive. 7-Oxo-DhA did not alter the spectrum of DitQ at concentrations up to $25 \mu\text{M}$, indicating that it is not a substrate for DitQ. The same assay was used to investigate binding of abietane diterpenoids to DitU; however, none of the compounds yielded definite binding spectra.

In vitro activity assays. To investigate the catalytic activity of DitQ and DitU, in vitro P450 activity assays were conducted. A crude lysate of *E. coli* expressing DitQ was combined with BphG, DitA3, NADH, and either AbA, DhA, PaA, or 7-oxo-DhA. BphG was purified from *C. testosteroni* and is the ferredoxin reductase component of biphenyl dioxygenase, a ring-hydroxylating Rieske-type oxygenase (15). DitA3 was purified from *P. abietaniphila* BKME-9 and is a ferredoxin. The *ditA3* gene (BxeC0638), encoding an LB400 homolog of DitA3_{BKME-9}, was up-regulated during growth of LB400 (38), suggesting that this ferredoxin might donate electrons to either DitQ or DitU or to both DitQ and DitU. GC-MS analysis of the reaction mixtures incubated with DhA revealed the formation of a single peak at a retention time of 1.11 relative to that of DhA. The mass spectrum of this peak was a clear match to that of diazomethane-derivatized 7-hydroxy-DhA. Inclusion of DitA3 was essential for the transformation. Incubation of expressed DitQ with other diterpenoids did not yield a detectable product. The same assays were conducted using a crude lysate of *E. coli* expressing DitU, but no products were detected.

DISCUSSION

Proteome versus transcriptome. The results of the current proteomic analysis agree well with the results of a previous transcriptomic analysis of LB400 grown on DhA (38). Most of the identified proteins with increased abundance on DhA (Table 3) were encoded by genes up-regulated on DhA; the only exception was BxeA1366, a gene outside the *dit* cluster. A high proportion of identified proteins with increased abundance on one or both abietane substrates (16 of 23 proteins) were encoded by genes in the 80-kb *dit* cluster. Similarly, the transcriptomic analysis indicated that 43 of 97 genes up-regulated on DhA are in the *dit* cluster. Importantly, both analyses support the conclusion that *ditQ* is the only P450 gene up-regulated on DhA.

DitQ and DitU. The genome of LB400 encodes six P450s (4). DitQ and DitU are the only P450s encoded in the *dit* cluster and share 61% amino acid sequence identity. By contrast, their levels of identity with the other four P450s are less than 25%. Our finding that DitQ is essential for LB400 to grow on DhA (Fig. 2) and to transform DhA (Fig. 3) differs somewhat from the findings of two previous studies in which disruption of *ditQ* homologs impaired but did not abolish growth on DhA of either *P. abietaniphila* BKME-9 (39) or *P. diterpeniphila* A19-6a (27). It is possible that these strains have additional P450s, possibly DitU homologs, that partially complement DitQ. In LB400, DitU is not expressed during growth on DhA, which may prevent complementation of DitQ.

While our proteomic analysis of LB400 indicated that both DitQ and DitU were expressed on AbA (Table 3), only DitU was required for growth on AbA (Fig. 2) and substantial removal of AbA (Fig. 3). Expression of *ditQ* on AbA appears to be an unnecessary consequence of its regulatory system. This pattern of expression is consistent with the results of a study

FIG. 3. Metabolite analysis of cell suspensions of LB400, DitQKO, and DitUKO incubated with AbA, DhA, and PaA. The scale for the filled symbols is on the left y axis, and the scale for the open symbols and the multiplication signs is on the right y axis. Symbols: $\blacklozenge$, AbA; $\blacksquare$ and $\blacktriangle$, DhA; $\blacktriangle$, PaA; $\circ$, 7-oxo-DhA; $\times$, 7-oxo-PaA. The data for wild-type strain LB400 are from reference 38. The data are from single experiments that were done in triplicate and in which consistent patterns of substrate removal and metabolite formation were observed. The final values were reproducible, but some differences in rates precluded averaging the data from independent experiments.

with BKME-9, in which a reporter construct demonstrated that *ditQ* is induced by AbA, DhA, and PaA (39).

Binding properties and catalytic activities of DitQ and DitU. The present study provides the first experimental evidence that DitQ transforms DhA to 7-hydroxy-DhA, which is the first catalytic activity demonstrated for a CYP226A subfamily P450. 7-Hydroxy-DhA is a metabolite in the bacterial (3) and fungal (17) degradation of DhA, and we previously proposed that DitQ_{BKME-9} transforms DhA to 7-hydroxy-DhA (39). Although DitQ_{LB400} shares 71% amino acid identity with its ortholog DitQ_{BKME-9}, the two proteins have distinct substrate-binding properties. These P450s bind DhA with comparable affinities (the K_d values are 0.98 ± 0.01 μ M [Fig. 4] and 0.43 ± 0.03 μ M [39], respectively), but only DitQ_{LB400} showed PaA binding. DitQ_{LB400} failed to transform PaA in our assay, but we cannot rule out the possibility of *in vivo* transformation, because the *in vitro* assay may have been limited by suboptimal conditions, including the use of a nonnative electron transport system. Similarly, we cannot rule out the possibility that DitQ_{LB400} binds AbA, since AbA perturbed the enzyme's spectrum.

The proteomic (Table 3) and gene disruption (Fig. 2 and 3)

analyses indicated that DitU is involved in the catabolism of AbA. However, the DitU substrate-binding assays and *in vitro* activity assays were inconclusive. Despite the lack of clear binding spectra, it is still possible that AbA or one of the other compounds tested binds to DitU, as substrates do not always yield type I binding spectra for P450s. Thus, neither deoxycorticosterone (36) nor morpholine (35) induces binding spectra in the P450s that transform them. As noted above, a suboptimal assay also may explain our failure to detect *in vitro* DitU activity. Alternatively, DitU may transform a catabolite of AbA.

Initial steps of diterpenoid catabolism. Several lines of evidence demonstrate that both DitU and DitQ are involved in the conversion by LB400 of abietane diterpenoids to the common intermediate 7-oxo-DhA (Fig. 5), the substrate for the ring-hydroxylating dioxygenase, DitA (23). The results unambiguously demonstrate that DitQ_{LB400} binds DhA and transforms it to 7-hydroxy-DhA (Fig. 2 to 4). The latter compound is presumably oxidized to 7-oxo-DhA by an unidentified dehydrogenase. This pathway for DhA is consistent with studies of BKME-9 (22, 39).

The present study provides evidence for a previously unrec-

FIG. 4. Binding spectra of DitQ and DhA or PaA. (A) DhA binding to DitQ. The data points indicate the difference in absorbance between 387 and 421 nm caused by increasing DhA concentrations. The curve represents a best fit of the binding equation (5) to the data, in which K_d is $0.98 \pm 0.01 \mu\text{M}$ and the maximum change in absorbance is $1.16 \times 10^{-1} \pm 6.55 \times 10^{-3}$. (Inset) UV-visible difference spectra for DitQ with increasing concentrations of DhA. (B) PaA binding to DitQ: UV-visible difference spectra of DitQ with increasing concentrations of PaA. Because an isosbestic point was not observed, PaA data could not be fit to the binding equation. The arrows indicate increases in the amplitude of the maximum or minimum absorbance caused by increasing the concentration of DhA or PaA.

ognized route for AbA degradation that requires the activity of DitU and involves oxidation of C-7 prior to aromatization of the C ring (Fig. 5). This route is consistent with production of 7-oxo-PaA in cell suspensions with AbA (Fig. 3). Previous studies of BKME-9 suggested that AbA is catabolized via DhA (22, 23). However, the latter route was not supported by the data obtained after disruption of *ditQ* in BKME-9, which severely limited growth on DhA but had little effect on growth on AbA (39). Other workers previously suggested that biotransformation of AbA can yield 7-hydroxy-AbA (7) or 7,8-epoxy-AbA (31). Also, fungal biotransformation of pimaradiene diterpenes, containing a B ring structure with a 7,8 double bond similar to AbA, led mainly to epoxides with the 7,8

FIG. 5. Proposed convergent pathway for abietane diterpenoid degradation by LB400, indicating proposed transformations catalyzed by oxygenases.

double bond plus 7-oxo derivatives (12, 13). Fraga et al. (12, 13) suggested that the 7,8-epoxide rearranged to the 7-ketone by opening of the oxirane ring. This mechanism would explain the production of 7-oxo-PaA from AbA in this study, which might be catalyzed by DitU. The additional accumulation of 7-oxo-DhA in cell suspensions with AbA could result from aromatization of ring C by an enzyme not identified yet. This proposed pathway is consistent with the results previously obtained with BKME-9 (22, 23, 39).

Our data strongly suggest two distinct routes for PaA catabolism in LB400 (Fig. 5). The first route involves aromatization of PaA to DhA and is consistent with the formation of DhA observed in cell suspensions incubated with PaA (Fig. 3). This aromatization of ring C of PaA is analogous to that proposed above for 7-oxo-PaA and might be catalyzed by the same enzyme. The DhA formed is presumably degraded as described above. The second proposed route involves hydroxylation of PaA to 7-hydroxy-PaA, which is further degraded via 7-oxo-PaA and subsequent intermediates described above. The second route is consistent with formation of 7-oxo-PaA in cell suspensions incubated with PaA (Fig. 3). It thus appears that 7-oxo-PaA is a point of convergence for AbA catabolism and PaA catabolism.

The involvement of DitQ and DitU in PaA catabolism has not been fully resolved. The partially impaired growth of DitQKO and DitUKO on PaA (Fig. 2) indicates that both P450s are likely involved in PaA catabolism. The DhA formed from PaA is presumably hydroxylated by DitQ. This role for DitQ is supported by the accumulation of DhA when DitQKO was incubated with PaA, but we cannot exclude the possibility that DitU also catalyzes this hydroxylation. The dramatic loss of PaA removal activity in DitUKO strongly suggests that DitU is primarily responsible for hydroxylation of PaA, but production of traces of 7-oxo-PaA by DitUKO suggests that DitQ may also have some capacity for this hydroxylation, consistent with binding of PaA to DitQ (Fig. 4B). Overall, DitU appears to play a more significant role than DitQ in PaA catabolism. However, further studies are required to define the roles of the P450s and other enzymes in PaA catabolism.

Electron transport system. The present study supports and expands our previous suggestion (38) that a multicomponent electron transport system is shared by three *dit*-encoded oxygenases, the P450s DitQ and DitU and the ring-hydroxylating dioxygenase DitA. BxeC0579 is the only ferredoxin reductase gene in the *dit* cluster of LB400 and is the only such gene up-regulated during growth on DhA (38). The *dit* cluster encodes two ferredoxins (38). The first ferredoxin, encoded by BxeC0601, is a typical plant-type 2Fe-2S ferredoxin. The second ferredoxin, encoded by *ditA3*, shares 60% amino acid sequence identity with the 3Fe-4S ferredoxin DitA3_{BKME-9} (6). DitA3_{LB400} and DitA3_{BKME-9} are 100% identical over regions containing Fe-S cluster ligands. A BKME-9 *ditA3* disruption mutant was unable to grow on DhA but did transform DhA to 7-oxo-DhA (22), indicating that in BKME-9, DitA3 is essential for DitA activity but not for DitQ activity. The current study demonstrates that DitA3_{BKME-9} can function with DitQ_{LB400} in vitro, suggesting that DitA3_{LB400} may function with DitQ_{LB400} in vivo. In LB400, it is possible that the 2Fe-2S ferredoxin encoded by BxeC0601 also functions with DitQ. BxeC0601 is located two open reading frames downstream of *ditQ* and is up-regulated during growth on DhA. If BKME-9 also has such a 2Fe-2S ferredoxin, this might explain why DitA3 is not essential for DitQ activity in this organism. Overall, there appears to be an overlapping system of electron transfer involving one ferredoxin reductase, two ferredoxins (3Fe-4S and 2Fe-2S), and the three oxygenases. This organization is reminiscent of the organization in *Spingomonas wittichii* RW1, in which two ferredoxins transfer electrons to a single dioxygenase (2), and the organization in *Ralstonia* sp. strain U2, in which a single reductase and ferredoxin transfer electrons to two oxygenases (43). To determine the compatibilities of the various LB400 proteins, further study with purified electron transfer and catalytic components is needed.

CYP226 family. Using available databases, we identified seven genes putatively encoding P450s belonging to the CYP226A subfamily (28). These P450s all share greater than 44% amino acid sequence identity. In addition to DitQ_{LB400} (CYP226A1) and DitU_{LB400} (CYP226A2), two other proteins are from bacteria known to grow on abietane diterpenoids as a sole source of carbon and energy: DitQ_{BKME-9} and TdtD (CYP226A3) from *P. diterpeniphila* A19-6a (27). Another two proteins are encoded by a putative *dit* cluster (38) in the recently sequenced genome of *Pseudomonas aeruginosa* 2192

(Broad Institute of Harvard and Massachusetts Institute of Technology; <http://www.broad.mit.edu>) and appear to be orthologs of DitQ_{LB400} and DitU_{LB400}. Other complete genomes with *dit* clusters were not found. The seventh CYP226A member, having 44 to 48% identity with the other members, is encoded by the draft genome sequence of *Caulobacter* sp. strain K31 (U.S. Department of Energy Joint Genome Institute; <http://www.jgi.doe.gov/>). CYP226A genes were also identified in the Sargasso Sea metagenome (41). Demonstration that divergent members of the CYP226A P450 subfamily are involved in diterpenoid catabolism would allow prediction of other abietane diterpenoid-degrading strains based on the presence of CYP226A homologs.

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