ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 2004, p. 3530–3535 Vol. 48, No. 9
0066-4804/04/$08.00+0 DOI: 10.1128/AAC.48.9.3530–3535.2004

Terbinafine Resistance Mediated by Salicylate 1-Monooxygenase in *Aspergillus nidulans*

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Received 6 February 2004/Returned for modification 21 March 2004/Accepted 19 May 2004

Resistance to antifungal agents is a recurring and growing problem among patients with systemic fungal infections. UV-induced *Aspergillus nidulans* mutants resistant to terbinafine have been identified, and we report here the characterization of one such gene. A sib-selected, 6.6-kb genomic DNA fragment encodes a salicylate 1-monooxygenase (*salA*), and a fatty acid synthase subunit (*fasC*) confers terbinafine resistance upon transformation of a sensitive strain. Subfragments carrying *salA* but not *fasC* confer terbinafine resistance. *salA* is present as a single-copy gene on chromosome VI and encodes a protein of 473 amino acids that is homologous to salicylate 1-monooxygenase, a well-characterized naphthalene-degrading enzyme in bacteria. *salA* transcript accumulation analysis showed terbinafine-dependent induction in the wild type and the UV-induced mutant Terb7, as well as overexpression in a strain containing the *salA* subgenomic DNA fragment, probably due to the multicopy effect caused by the transformation event. Additional naphthalene degradation enzyme-coding genes are present in fungal genomes, suggesting that resistance could follow degradation of the naphthalene ring contained in terbinafine.

The incidence of fungal infections has increased in the last 20 years, primarily because of the increase in the number of immunocompromised patients due to AIDS, malnutrition, the indiscriminate use of antibiotics, chemotherapy, and artificial immunosuppressive treatment in organ transplants (17). In addition, the widespread use of antifungal agents has led to clinical resistance. Thus, insights into molecular and genetic mechanisms involved in resistance are needed to define treatment strategies and to design new antifungal drugs.

Terbinafine is a generic antifungal agent used to treat superficial mycoses such as dermatophyte onychomycosis, dermatomycoses, tinea, and piedra (1, 6, 9, 20). Terbinafine interferes with ergosterol biosynthesis by inhibiting a membrane-bound squalene epoxidase (31). Inhibition of squalene epoxidase results in ergosterol deficiency with the accumulation of squalene, which may be responsible for the observed "in vitro" fungicidal activity (11, 26).

Besides the fact that fungi resistant to terbinafine have been consistently isolated, little is known about the molecular mechanisms associated with resistance (14, 22, 30, 38). In one case, a modified squalene epoxidase with reduced affinity for terbinafine conferred terbinafine resistance to *Nectria haematococca* mutants (15) and, in another, a *Candida albicans* strain was resistant to terbinafine due to activation of the multidrug efflux transporter *CDR2* (35). Also, a *C. albicans* mutant carrying *CDR1* deletion resulted in azoles and terbinafine hypersusceptibility (36).

We report here the involvement of naphthalene degradation as a possible mechanism of terbinafine resistance in *Aspergillus nidulans*. Transformation experiments demonstrate that terbinafine resistance is mediated through the overexpression of *salA*, a gene that encodes a salicylate 1-monooxygenase. In *Pseudomonas putida*, salicylate 1-monooxygenase catalyzes the formation of catechol from salicylate, a required intermediate of naphthalene degradation (2). Thus, terbinafine, which has a naphthalene nucleus in its chemical structure, may be a substrate for a salicylate-like part of an aromatic compound degradation pathway in *A. nidulans*.

MATERIALS AND METHODS

Strains, media, and culture conditions. *A. nidulans* was cultivated at 37°C in complete medium (CM) or minimal medium (MM) (27). The following strains were used: the wild type, FGSCA26 (*biA1 veA1*), the terbinafine-resistant strains Terb7 (*pabaA1 tebA7 veA1*) (30) and Terb7A (*acrA1 tebA7 veA1*), the transformation recipient strains GR5 (*pyroA4 pyrG89 wA3 veA1*) and RPA26 (*biA1 ΔargB::trpC801 trpC801 veA1*). The mutant alleles were as follows: *wA3*, white conidia; *tebA7* and *acrA1*, terbinafine and acriflavin resistance, respectively, and the auxotrophic markers; *ΔargB::trpC801*, *biA1*, *pabaA1*, *pyrG89*, and *pyroA4* for arginine, biotin, *p*-aminobenzoic acid, uracil, and pyridoxine, respectively. Terbinafine [(E)-*N*-(6,6-dimethyl-2-hepten-4-yn-yl)-*N*-methyl-1-naphthalene methanamine] was from Sandoz AG, dissolved in dimethyl sulfoxide, and added to solid or liquid medium. An agar dilution assay was used for terbinafine susceptibility testing. A 100-μl suspension of the conidia of each strain (10^7 conidia per ml) was inoculated into solid-CM dishes with various concentrations of terbinafine, followed by incubation at 37°C for 3 days. The MIC corresponds to the lowest concentration of the drug at which there is no macroscopic growth. All assays were carried out in triplicate (8). *Escherichia coli* strain DH5α (12) or XL1-Blue (Stratagene) was used for the propagation of plasmid DNA, which was cultivated at 37°C in Luria-Bertani broth amended with 50 μg of ampicillin/ml.

Molecular nucleic acid manipulation techniques and sequencing. DNA manipulation and cloning procedures were performed as described either by Sambrook et al. (33) or by the supplier of enzymes and nucleic acid reagents. Shotgun subcloning, random clone sampling (Fig. 1C), and nucleotide sequence were determined by the dideoxynucleotide chain termination method (34) using BigDye terminator cycle sequencing (Perkin-Elmer) in an ABI Prism 377 DNA sequencer. DNA sequence data files were assembled by using the Phred, Phrap, and Consed packages (7, 10).

The original terbinafine-resistant plasmid was subcloned into smaller frag-

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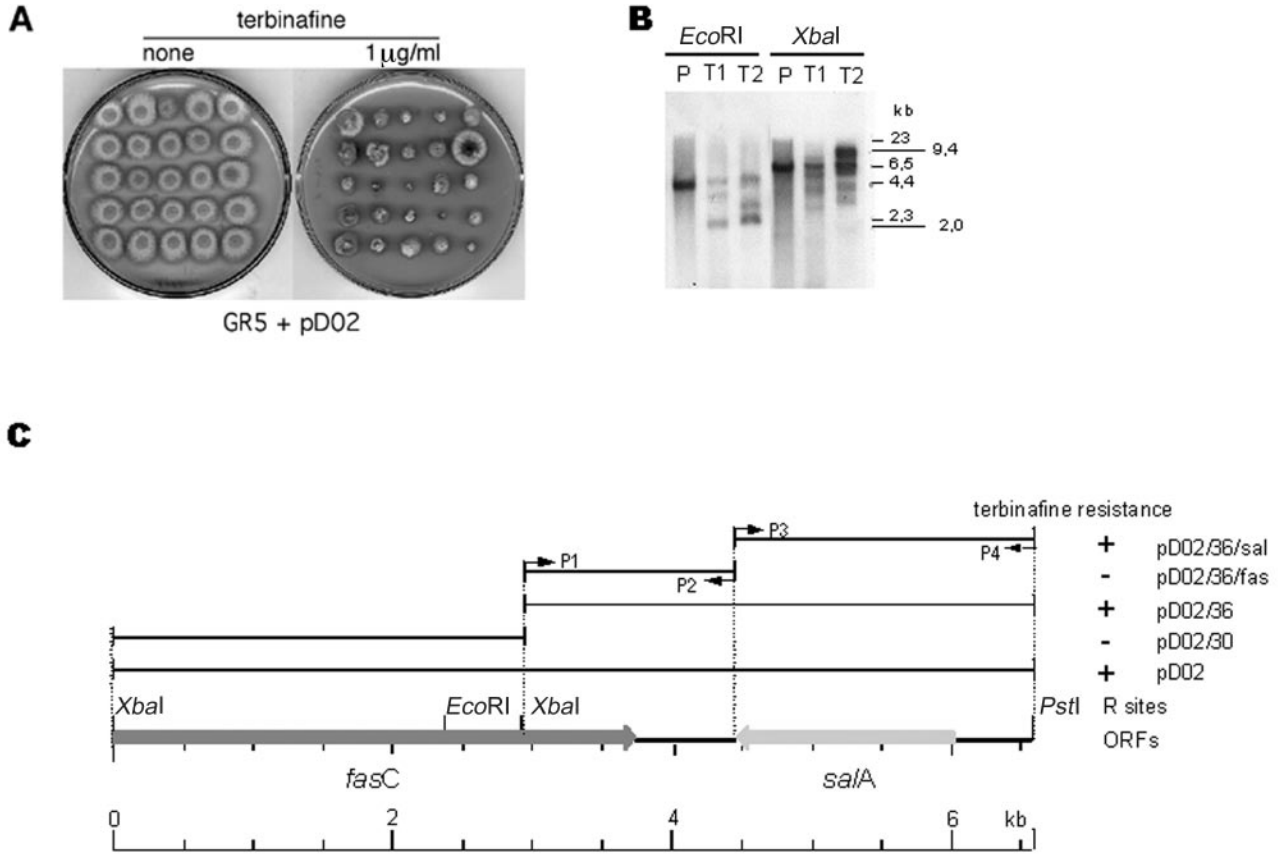

FIG. 1. The *salA* gene confers terbinafine resistance in *A. nidulans*. (A) Identification of a subgenomic DNA fragment (pD02) that confers terbinafine resistance. Strain GR5 was transformed with plasmid pD02, which was shown in a sib selection experiment to induce terbinafine resistance. Colonies are independent transformants randomly selected after transformation with pD02 and grown on medium with or without 1 μg of terbinafine/ml. (B) Southern blot analysis of the genomic DNA of *A. nidulans*. Genomic DNA from GR5 (lanes P) and two pD02 transformant strains (lanes T1 and T2) were digested with EcoRI or XbaI, size fractionated through a 1.0% agarose gel, and blotted as described in Materials and Methods. The probe used was the *salA* fragment obtained from pD02/36/sal contained in pGEM-T Easy vector. Size markers (in kilobases) are indicated at right of the blot. (C) Salicylate 1-monooxygenase confers terbinafine resistance. Plasmid pD02 was subcloned, and the genomic DNA sequence of the region was determined. Two ORFs, *fasC* and *salA*, were identified. DNA-mediated transformation-dependent resistance analyses showed that only fragments that contain *salA* are terbinafine resistant. Primers P1 to P4 were used to construct the subclones pD02/36/sal and pD02/36/fas.

ments by cleaving pD02 fragment with XbaI and PstI, resulting in 3.3-kb XbaI-PstI and 3.1-kb XbaI fragments. Further subcloning, isolating the *fasC* and *salA* open reading frames (ORFs), was pursued by PCR amplification (21) of the indicated regions on plasmid pD02/36 (shown in Fig. 1) with specific primers (see below). PCR product were cloned and propagated in *E. coli* with the pGEM-T Easy vector (Promega).

The following oligonucleotides were used: pUCM13 forward, 5′-GTAAAACGACGGCCAGT-3′; pUCM13 reverse, 5′-CAGGAAACAGCTATGAC-3′; P1 (fas1-5), 5′-GTGTTTTAGCATTCTCGCC-3′; P2 (Anfatty16), 5′-AGGATCACGACATTCACTTG-3′; P3 (ansalA4), 5′-CTCCAAGTCGCCCCCAATC-3′ and P4 (sal1-5), 5′-CACGGGACGGGAACACCATC-3′

Library construction. Total DNA from the Terb7A was extracted by the method of Reader and Broda (29) from a 12-h-old liquid culture shaken at 37°C after the inoculation of 10^7 conidia per ml. Partially Sau3AI-digested DNA fragments were ligated to the pRG4 shuttle vector (19), which confers uracil auxotropy when integrated into the genome of *A. nidulans* strain GR5, and transformed into *E. coli* XL1-Blue by electroporation. A total of 17,856 clones with an average insert size of 6.5 kb were recovered, and 7,680 of them were transformed into strain GR5 (~1.6-fold genome coverage).

Transformation and sib selection. Transformation experiments were performed as previously described (40), except that the protoplasts were made with Glucanex (Novo Nordisk) rather than with Novozym. A sib selection screen with modifications (33) was used in order to rescue a recombinant plasmid bearing the gene that confers terbinafine resistance. The approach is based on the concept of dividing a large genomic library into a manageable number of pools, each consisting of 960 clones (10 96-well plates). These pools are then tested for the ability to confer terbinafine resistance in 1 μg of terbinafine/ml. After a pool is identified, it is subdivided into successively smaller and smaller pools, until a unique recombinant plasmid is isolated. Once an individual plasmid was identified, the cloned insert was sequenced and characterized.

Southern and Northern blot hybridizations. Southern blot analysis was performed by standard techniques (33). For Northern blots, total RNA isolation of 1 to 2 g of lyophilized ground mycelium was obtained by using the TRIzol method (4) and isolated according to manufacturer recommendations (Gibco-BRL). Northern blot analysis was performed as described elsewhere (42). Briefly, 10 μg of total RNA was separated on 1.1% agarose containing 3% formaldehyde and blotted onto nylon membranes (Hybond N^+; Amersham Pharmacia Biotech). [^{32}P]CTP-labeled probes were used to hybridize the membrane with modified Church buffer (5, 42) at 65°C for 16 to 20 h. Blots were washed and exposed to film and developed according to the method of Sambrook et al. (33).

The GenBank accession numbers for *salA* and *fasC* are AF316427 and AY120937, respectively.

RESULTS

Isolation of a gene that alters *A. nidulans* susceptibility to terbinafine. To better understand terbinafine resistance, we screened for recombinant plasmids able to turn sensitive

TABLE 1. Salicylate monooxygenase *salA* genes in filamentous fungi

Analysis type and ORF or locus	Organism	BLASTp or TBLASTn analysis[a]						
		P	Bit score	Identities		Positives		No. of gaps (%)
				aa or bp	%	aa or bp	%	
BLASTp								
AN3382.1	*Aspergillus nidulans*	$1E^{+00}$	878	435/473	91	435/473	91	0
MG10012.3	*Magnaporthe grisea*	$1E^{-70}$	263	159/439	36	245/439	55	6
NCU07598.1	*Neurospora crassa*	$1E^{-54}$	209	136/438	31	214/438	48	12
FG03657.1	*Fusarium graminearum*	$2E^{-52}$	202	131/407	32	206/407	50	9
nahG	*Pseudomonas putida*	$2E^{-27}$	122	114/441	25	197/441	44	17
TBLASTn								
1.190 (sfd 16)	*Ustilago maydis*	$2E^{-25}$	113	118/437	27	183/437	41	4
1.143 (sfd 7)	*Coprinus cinereus*	$3E^{-08}$	57	57/218	26	89/218	40	20

[a] BLASTp, the *A. nidulans salA* amino acid sequence was compared with the predicted ORFs of fully sequenced genomes; TBLASTn, the *A. nidulans salA* amino acid sequence was compared with the translated genomic nucleotide sequence. BLASTp identities are expressed as amino acids (aa); TBLASTn identities are expressed as base pairs. Identity is the extent to which two amino acid sequences are invariant, expressed as the number identical amino acids/total amino acids in the protein. Positive is the extent to which protein sequences are related, expressed as the number of similar amino acids/total amino acids in the protein.

strains resistant that were made from genomic DNA of strain TerbA7, whose resistance to terbinafine had been genetically defined previously (30). We isolated a recombinant plasmid, pD02, which was able to change GR5 strain from being sensitive to being resistant to terbinafine. To confirm that the complementing activity did not arise from spontaneous reversions, the plasmid was retransformed into GR5.

Figure 1A shows that transformation of plasmid pD02 into GR5 results in multiple-level terbinafine-resistant strains. Figure 1C shows the subcloning, a physical map of the 6.6-kb chromosomal region contained in pD02, and mapping of the terbinafine resistance to a single ORF. The genomic DNA sequence reveals two ORFs: *fasC*, encoding the beta subunit of the *A. nidulans* fatty acid synthase (3), and *salA*, a salicylate 1-monooxygenase-like protein known to be associated with naphthalene degradation in *Pseudomonas stutzeri* AN10 (2). The subclones pD02/36 and pD02/30 were transformed into *A. nidulans*, and only pD02/36 was able to confer terbinafine resistance (Fig. 1C). Two additional genomic subregions were created by using PCR-amplified fragments and maintained in pGEM-T. Only the *salA*-containing fragment was capable to confer terbinafine resistance (Fig. 1C).

Genomic characterization and deletion of the *salA* locus. The *salA* ORF is contained by 1,521 bp encoding a 473-amino-acid residue protein (GenBank accession number AF316427) interrupted by one putative intron (positions 578 and 680) deduced from consensus sequence of fungi. The predicted peptide encodes a salicylate 1-monooxygenase, an enzyme involved in naphthalene degradation. Table 1 shows that all fully sequenced fungal genomes to date contain at least one predicted protein product with high degree of homology to *A. nidulans salA*.

Restriction enzyme Southern genomic mapping indicates that *salA* is present as a single copy in the genome (Fig. 1B, lanes P). Hybridization of a radioactively labeled *salA* DNA fragment with blotted pulsed-field electrophoresis separated chromosomes (data not shown) and the minimal tiling cosmid collection (28) identify a single cosmid (data not shown), suggesting that *salA* is physically located on chromosome VI. The profile of Southern blot hybridization was consistent with *salA* being a single-copy gene suitable for targeted gene disruption experiments. The inactivation of gene *salA* in strain RPA26 did not change the sensitivity of the fungus to terbinafine, i.e., the relative terbinafine resistance of wild-type, RPA26,

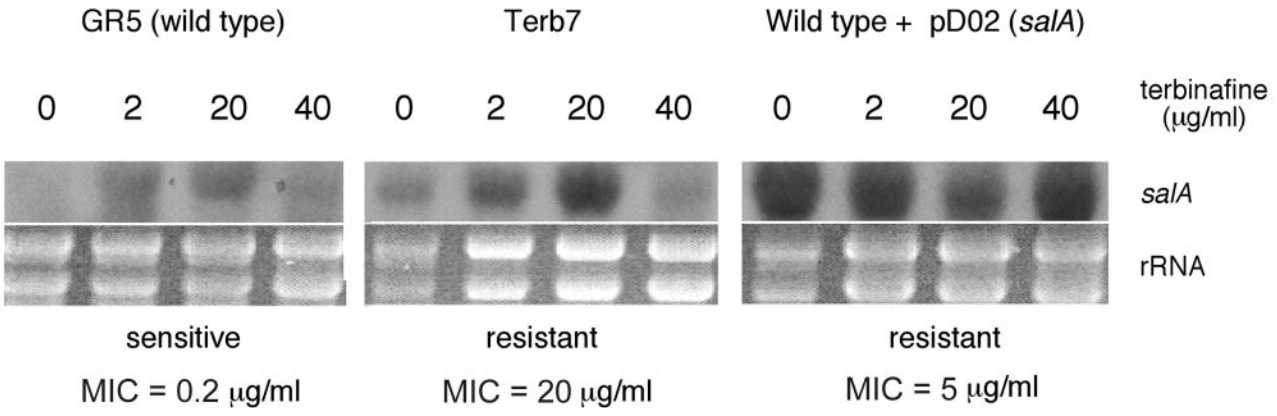

FIG. 2. Terbinafine-dependent and overexpressed *salA* transcript accumulation in wild-type and mutant strains. *salA* transcript accumulation analysis in response to various concentrations of terbinafine in wild type, Terb7, and a pD02 transformant was carried out.

and Δ*salA* mutant strains, expressed as the terbinafine MIC, was 0.2 μg/ml.

Terbinafine-dependent *salA* transcript accumulation. The mutation in Terb7, which confers semidominant terbinafine resistance, has been mapped to chromosome IV (30). Thus, the pD02 DNA insert, which maps to chromosome VI and encodes SalA, mediates terbinafine resistance, apparently independently of the *tebA7* mutation.

Figure 2 shows terbinafine-dependent, *salA* transcript accumulation in a wild-type strain, a pD02-transformant and Terb7 (Fig. 1A and C). Vegetative mycelia were incubated in liquid shake-flask cultures with the indicated amounts of terbinafine and total RNA extracted, separated by gel electrophoresis, blotted onto nylon membranes, and hybridized with a radioactively labeled *salA* probe.

The presence of terbinafine promotes accumulation *salA* transcript, which appears to be a transcriptionally regulated event in the wild-type (sensitive) and Terb7 (resistant) strains but not in a pD02 transformant (resistant). Figure 2 shows that the *salA* transcript accumulates when terbinafine was added to the culture medium at 2 and 20 μg/ml in a wild-type strain and Terb7; however, the expression of *salA* is terbinafine independent in a pD02 transformant at the concentrations assayed (2 to 40 μg/ml). Interestingly, *salA* transcript does not accumulate when terbinafine is 40 μg/ml, except for the pD02 transformant (terbinafine independent) (Fig. 2).

Other fungal genes associated with naphthalene degradation in filamentous fungi. One possible explanation for the observations described in Fig. 2 relies on the notion that the mutation in strain Terb7 targets a regulatory gene located on chromosome IV, which affects the expression of *salA*, located on chromosome VI. In pD02 transformants overexpression of the salicylate 1-monooxygenase transcript causes terbinafine resistance and is apparently independent of *tebA7*. Terbinafine contains a naphthalene ring, a possible substrate for the *A. nidulans* SalA. Naphthalene degradation as is typically observed in bacteria involves a series of enzymes and has not yet been described in fungi.

Table 2 gives the homology scores of the closest fungal homolog to microorganism genes involved in naphthalene degradation. Genes that are present in *A. nidulans* and other filamentous fungi are as follows: 2-hydroxychromene-2-carboxylate isomerase, $P = 4e^{-40}$ in *A. nidulans* only; salicylaldehyde dehydrogenase; $P = 6e^{-92}$, $1e^{-91}$, $2e^{-96}$, and $8e^{-94}$; salicylate 1-monooxygenase, $P = 2e^{-63}$, $1e^{-32}$, $6e^{-42}$, and $8e^{-53}$; and 2-hydroxymuconic semialdehyde dehydrogenase, $P = 9e^{-94}$, $4e^{-98}$, $2e^{-91}$, and $3e^{-90}$ in *A. nidulans*, *Magnaporthe grisea*, *Neurospora crassa*, and *Fusarium graminearum*, respectively. Genes that could not be found through homology matching fungal genome database entries include 1,2-dihydroxy-naphthalene dioxygenase, 2-hydroxychromene-2-carboxylate isomerase, and 4-hydroxy-2-oxovalerate aldolase.

TABLE 2. Naphthalene degradation genes in filamentous fungi

Function	Homology search source			Homology target genome									
	Organism	Protein	*Aspergillus nidulans*			*Magnaporthe grisea*		*Neurospora crassa*		*Fusarium graminearum*			
			ORF	Bit score	*P*	Bit score	*P*	Bit score	*P*	Bit score	*P*		
Naphthalene 1,2-dioxygenase	*Pseudomonas* sp. strain ND6	AAP44248	AN8998	122	9E−29	111	3E−25	110	3E−25	119	2E−27		
Tetrahydroxynaphthalene reductase	*Neurospora crassa*	EAA29497	AN1886	82	1E−16	81	3E−16	92	2E−19	84	3E−17		
1,2-Dihydroxy-naphthalene dioxygenase					No hit		No hit		No hit		No hit		
2-Hydroxychromene-2-carboxylate isomerase	*Rhodopseudomonas palustris*	ZP_00010435	AN6936	159	4E−40		No hit		No hit		No hit		
	Pseudomonas syringae	NP_793938			No hit	68	2E−12	96	5E−21	99	1E−21		
Salicylaldehyde dehydrogenase	*Sphingomonas* sp.	AAD45415	AN1689	333	6E-92	332	1E−91	327	2E−96	340	8E−94		
	Pseudomonas sp. strain ND6	AAP44246	AN4050	330	4E−91	314	4E−86	313	8E−86	307	4E−84		
Salicylate 1-monooxygenase	*Pseudomonas stutzeri*	AAD02146	AN7382	238	2E−63	136	1E−32	167	6E−42	203	8E−53		
	Mesorhizobium loti	BAB53776	AN3569	140	5E−34	154	3E−38	128	2E−30	169	2E−42		
Catechol oxygenase	*Pseudomonas putida*	AAA66204	AN4532	130	6E−31	133	7E−32	141	2E−34	129	1E−90		
2-Hydroxymuconic semialdehyde dehydrogenase	*Pseudomonas putida*	S107722	AN9034	339	9E−94	353	4E−98	331	2E−91	328	3E−90		
4-Hydroxy-2-oxovalerate aldolase	*Novosphingobium aromaticivorans*	AAD04028	AN0080	140	4E−34		No hit		No hit		No hit		

DISCUSSION

Failure in treating fungal infections with terbinafine has been reported for patients infected with *Trichophyton rubrum* (22) and *C. albicans* (32). In *C. albicans*, multidrug efflux transporters have been implicated in resistance (35, 36). Thus, in order to obtain data that might contribute to a better under-

standing of antifungal resistance mechanisms, mutants resistant to terbinafine were isolated and characterized by classic genetic analysis (30). We constructed a genomic library of one such mutant, Terb7A, and through sib selection we identified clone pD02 (Fig. 1), which encodes two previously unreported genes: *salA*, which codes for a prokaryotic salicylate 1-monooxygenase (2), and the C-terminal portion of the *fasC* gene, which codes for the β subunit of the enzyme fatty acid synthase.

Subcloning of pD02 and creation of plasmids carrying the *fasC* (pD02/36fas)- or *salA* (pD02/36/sal)-encoding fragments of pD02 (see Fig. 1C) revealed that only fragments containing *salA* confer terbinafine resistance upon transformation into a sensitive *A. nidulans* strain. Bacterial salicylate 1-monooxygenase catalyzes the formation of catechol from salicylate, an intermediate from naphthalene degradation (2). Thus, terbinafine, which has a naphthalene nucleus in its chemical structure (23–25, 39), could be the substrate for a similar degradation pathway in fungi, generating salicylate or an analogous compound as substrate for the enzyme salicylate 1-monooxygenase in *A. nidulans*.

Most of the information about naphthalene metabolism has been obtained from bacteria such as *P. putida* (41), with which salicylate 1-monooxygenase is an extracellular flavoprotein that catalyzes conversion of salicylate to catechol by introduction of a hydroxyl group with concomitant removal of a carboxyl group (16). In addition, naphthalene degradation is a regulated process and involves *nahR*, a positive regulator of transcription induced by salicylate (37).

In fungi, aromatic compounds are initially converted into orthodiphenolic intermediates, e.g., catechol and protocatechuate, hydrolyzed between two hydroxyl groups and catabolized via the β-ketoadipate pathway, producing succinate and acetyl-coenzyme A (13).

Even though the genomic DNA library used in transformation experiments contained the *tebA7* allele, which is genetically mapped to chromosome IV, we found one terbinafine-resistance-conferring DNA fragment, which contains the *salA* gene, physically mapped to chromosome VI. The physical location of the *salA* DNA fragment was verified by three means: (i) by DNA-DNA hybridization to pulsed-field-gel-electrophoresis-separated chromosomes, (ii) hybridization to a single chromosome VI cosmid of the *A. nidulans* physical map (28), and (iii) the determination that *salA* is on chromosome VI of the fully sequenced genome *A. nidulans* database at the Whitehead Institute (Cambridge, Mass.). Thus, we hypothesize that an apparent multicopy effect of the *salA* gene observed by Southern analysis of the pD02-*salA* transformant strains (Fig. 1B) may confer resistance to terbinafine in a manner similar to the mutation in the *tebA7* gene, supporting the suggestion of Rocha et al. (30) that terbinafine resistance should involve multiple genes that appear to act within a genetically regulated circuit.

Northern blot experiments show (Fig. 2) increased *salA* transcript accumulation by terbinafine concentrations in wild-type and Terb7, indicating that *salA* transcript accumulation is affected by the presence of terbinafine and likely to be regulated by *tebA7*. Furthermore, in pD02 transformants, *salA* is expressed at high levels, independent of the presence of terbinafine, suggesting that salicylate or an intermediary degradation product is catalyzed more effectively in Terb7 and pD02 transformant than in the wild type. Finally, when *salA* is less abundant, levels of intracellular salicylate or an analogous compound may be more elevated. In *P. aeruginosa* membrane protein, synthesis associated with multiple drug resistance is suppressed by the presence of salicylate in the cell, rendering cells sensitive to different drugs (18).

The inalterable terbinafine sensitivity exhibited by the null *salA* allele supports the idea that resistance is due to an altered accumulation of *salA* transcript and the consequent increase in salicylate 1-monooxygenase and degradation of the naphthalene ring of terbinafine in the pD02 transformant compared to the wild-type strain.

ACKNOWLEDGMENTS

This study was supported by grants from FAPESP, CNPq, CAPES, and FAEPA.

We thank R. A. P. Ferreira, M. Mazucatto, A. C. Crescenço, and L. N. Oliveira for technical support.

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