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A *Botrytis cinerea* putative 3-keto reductase gene (*ERG27*) that is homologous to the mammalian 17β-hydroxysteroid dehydrogenase type 7 gene (17β-HSD7)

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Abstract

Botrytis cinerea (anamorph of *Botryotinia fuckeliana*) is a filamentous ascomycete that causes grey mould on grapevine. We had previously described two distinct populations, named HydR1 and non-HydR1, that comprise two distinct genetic entities based on genetic polymorphism, natural resistance towards the fungicide hydroxyanilide fenhexamid, and vegetative incompatibility between them. Here, we used PCR to isolate the 3-keto reductase gene *ERG27* by virtue of sequence homology with *Saccharomyces cerevisiae ERG27*. The gene product was longer than the yeast's enzyme but possessed the main characteristic features of reductases. It displayed striking homology with mammalian 17β-HSD7, therefore confirming the hypothesis of a common function between Erg27p like protein and 17β-HSD7 in sterol biosynthesis (i.e. cholesterol, ergosterol). On the other hand, we analysed the polymorphism of the *B. cinerea* gene product and found a dozen of amino-acid differences between strains of HydR1 and non-HydR1 types that could underlie HydR1 natural resistance to fenhexamid. First, this polymorphism analysis showed that HydR1 strains form a homogeneous group distinct from the non-HydR1 group of strains. These results support our hypothesis that HydR1 and non-HydR1 strains constitute two different species. Second, Erg27p like protein sequence analysis showed that a high resistant phenotype to fenhexamid, HydR3, found in treated populations of non-HydR1 strains, had two mutations (usually found in mammalian 17β-HSD7) that could be useful as population markers.

Introduction

Botrytis cinerea (anamorph of *Botryotinia fuckeliana*) is an ascomycete that causes grey mould on economically important crops, such as vegetables, ornamentals and fruits. Because of the wide range of hosts and tissues that it attacks, *B. cinerea* has been thought to be unspecialized, unlike the other species of its genus. However, molecular analyses using RFLP markers showed that *B. cinerea* exhibits a great genetic diversity relying upon permanent genetic recombination events and further analyses, based on the presence or the absence of two transposable elements (*Boty* and *Flipper*), ended in the description of two sibling sympatric populations, named *transposa* and *vacuma* (Giraud et al., 1997, 1999). Some strains, mainly encountered among the *vacuma* population and designated HydR1, are found to be naturally resistant to fenhexamid. Such HydR1 strains showed an increased sensitivity to 14α-demethylase inhibitors (DMIs) (Leroux et al., 1999). Because mutations at the target gene level (*CYP51*) could underlie the observed increased sensitivity of HydR1 strains to DMIs, we performed systematic sequencing of the *CYP51* gene from various *B. cinerea* strains (Albertini et al., 2002). These investigations showed, that HydR1 *CYP51* had two specific expressed mutations and that the general polymorphism at this particular locus was significantly high. Further analyses revealed that *CYP51* polymorphism did not discriminate between *transposa* and *vacuma* strains but did distinguish between HydR1 and non-HydR1 ones (Albertini et al., 2002). These data,

combined with the existence of morphological differences and somatic incompatibility between HydR1 and non-HydR1 strains, suggested these two groups comprise two distinct genetic entities that might even be considered to be two different species (Albertini et al., 2002; Fournier et al., 2003). Two more fenhexamid-resistant phenotypes, HydR2 and HydR3, have been described in fenhexamid treated populations (Leroux et al., 2002a). HydR2 and HydR3 strains were found in *transposa* populations, but did not exhibit increased sensitivity towards DMIs and were distinguished from one another by *in vitro* testing of the effects of fenhexamid on germ-tube elongation.

The role of fenhexamid in altering sterol biosynthesis has been shown to be due to a novel mode of action. Fenhexamid affects sterol C-4 demethylation through inhibition of one of the three microsomal enzymes involved in the C-4 demethylation process: the 3-keto reductase enzyme (Debieu et al., 2001). In *Saccharomyces cerevisiae*, the *ERG27* gene that encodes 3-keto reductase has been cloned by complementation in a 3-keto reductase mutant which was unable to grow without the addition of sterols such as ergosterol or cholesterol. Disruption of *ERG27* in a wild-type strain produced the same 3-keto reductase mutant phenotype, whereas integration of the *ERG27* wild-type allele restored cell growth in unsupplemented culture medium (Gachotte et al., 1999). In *B. cinerea,* we found that a cDNA expressed sequence tag (EST) presented, after conceptual translation, significant homology with the *S. cerevisiae* Erg27p enzyme. Therefore, we decided to clone the *B. cinerea ERG27* gene in order to establish whether there was a relationship between phenotypes of resistance or sensitivity to fenhexamid and the polymorphisms at this particular locus.

Materials and methods

Fungus, culture and phenotypes

Most of the strains of *B. cinerea* used were kindly supplied either by the Laboratoire de Phytopathologie et Méthodologie de la Détection (INRA, 78026 Versailles Cédex), or by Bayer Crop Science (Germany). Strains of *Sclerotinia sclerotium* were isolated from stems of field grown rape (Table 1). They were maintained

Table 1. Phenotypic characterization of strains

Strain number[a]	Host	Organ	Location	Transp. elements[b]	Phenotype/fungicide[c,d]			
				Boty, *Flipper*	Hyd	Imi	Ben	DMIs
Botrytis cinerea								
971	Grape	Berries	Trepail	*flipper only*	R1	S	S	HS
780	Grape	Leaves	Plumecoq	*vacuma*	R1	S	S	HS
1258	Pea	Flowers	Plumecoq	*vacuma*	R1	S	R1	HS
1771	Grape	Berries	Germany	*transposa*	R2	S	S	S
1790	Vegetable	?	Japan	*transposa*	R2	S	R2	S
617	Grape	Leaves	Boursault	*transposa*	S	R	R1	S
154[e]	Grape	Berries	Champagne	*transposa*	R[e]	R[e]	R1	R[e]
L	Grape	Berries	Bordeaux	*transposa*	S	S	R1	S
T4	Tomato	?	Eyragues	*transposa*	S	S	S	S
1836	Grape	Berries	Venningen (Germany)	*transposa*	R3	S	S	S
1837	Grape	Berries	Venningen (Germany)	*transposa*	R3	S	S	S
Sclerotinia sclerotium								
72-4T	Rape	Stems	Lorraine		S	S	S	S
00-R2	Rape	Stems	Ile de France		S	S	R1	S
68-6	Rape	Stems	Lorraine		S	R	S	S
B	Rape	Stems	Ile de France		S	R	R1	S

[a]1771, 1790, 1836, 1837 are from Bayer Crop Science.
[b]Transposable elements: a strain which has both *Boty* and *Flipper* is *transposa*, a strain which lacks those elements is *vacuma*, a strain having *Flipper* as a single transposable element is called *flipper only*.
[c]S: sensitive; HS: hypersensitive; R: resistant, see Table 2.
[d]Hyd: hydroxyanilides like fenhexamid; Imi: dicarboximides as vinclozolin; Ben: benzimidazoles; DMIs: 14α-demethylase inhibitors like prochloraz.
[e]154 is a multidrug resistant strain with low levels of resistance towards fenhexamid, DMIs, anilinopyrimidines and dicarboximides (Chapeland et al., 1999).

on malt agar containing yeast extract (Leroux et al., 1999).

Detection of transposable elements *Boty* and *Flipper* in *B. cinerea* strains was achieved by dot blot analysis (Giraud et al., 1997).

Effects of fenhexamid upon germination of conidia and germ-tube elongation were studied by spreading conidia suspensions (2×10^5 ml^{-1}) on the surface of an agar (10 g glucose, 2 g K_2HPO_4, 2 g KH_2PO_4 and 12.5 g agar per litre) containing discriminatory fungicide concentrations. After incubation for 24 or 48 h in the dark, at 20 °C, the average length of germ-tubes was evaluated under the microscope. Effect of fungicides on mycelial growth were studied as described previously (Leroux et al., 1999) and from the dose-response curves, EC_{50} values (concentrations causing a 50% reduction in the mycelium growth rate) were calculated.

DNA extraction

DNA was extracted from freeze-dried mycelium of *B. cinerea* or *S. sclerotium* strains, grown in liquid medium at 18 °C, using a CTAB protocol (Albertini et al., 1999).

Identification of a partial ERG27 *sequence*

Taking the *S. cerevisiae ERG27* gene product sequence as a reference (GenBank accession number U53876.1), we performed a BLAST search of the protein database at NCBI. A cDNA fragment from a *B. cinerea* T4 EST cDNA library (EMBL accession number AL116217.1) was found to have, after conceptual translation, significant homology with part of the yeast 3-keto reductase enzyme. Therefore, we designed specific primers for PCR cloning of *ERG27* from the T4 strain using the genome walking procedure.

Genome walking

Genome walker libraries were constructed following manufacturer's instructions (Clontech laboratories Inc., USA) with freshly extracted DNA from strain T4 (*transposa*, HydS, see Table 1). Briefly, ~2.5 µg genomic DNA were digested to completion with one of the restriction enzymes *Dra*I, *Stu*I, *Eco*RV or *Pvu*II. Synthetic DNA adaptors were ligated to genomic DNA fragments to produce uncloned genomic libraries. Two PCR amplification steps were performed successively. A primary PCR with an outer adaptor primer AP1 provided in the kit and an outer gene specific primer, followed by a nested PCR with a nested adaptor primer AP2 from the kit and a nested specific primer (Figure 1A–C). To amplify upstream sequences of *ERG2*, we designed ERGPup for both primary and nested PCR which corresponds to amino-acid sequence YVVQPGIF in the antisense direction of the previously amplified gene fragment. Similarly, to amplify the downstream sequence of the

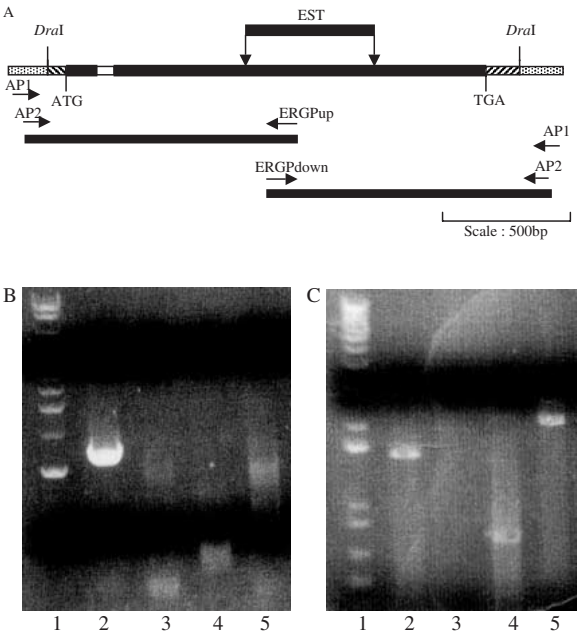

Figure 1. A. Schematic representation of *B. cinerea ERG27* like gene isolation by genome walking. The open reading frame is marked in black and is interrupted by a unique intron indicated as a white box. Sequences upstream and downstream of the ORF are shown with shaded boxes. Ligated synthetic adaptors are indicated by dotted boxes. Horizontal arrows below the *ERG27* gene indicate the different primers used in the genome walking procedure. Vertical arrows show the extent and position of the EST fragment. PCR products obtained by genome walking on *B. cinerea* T4 strain. After restriction enzyme treatment (*Dra*I: lanes 2B and 2C; *Eco*RV: lanes 3B and 3C; *Pvu*II: lanes 4B and 4C; *Stu*I: lanes 5B and 5C) DNA digested fragments were ligated to synthetic adaptors and PCR was performed in each case using a specific *ERG27* primer (ERGup or ERGdown) and a specific adaptor primer. Lanes 1B and 1C: DNA molecular weight markers. B. Upstream amplification of *ERG27* using ERGup specific primer gave a 1.2 kb product after *Dra*I digestion (lane 2). C. Downstream amplification of *ERG27* using ERGdown specific primer gave a 1 kb product after *Dra*I digestion (lane 2). These two *Dra*I overlapping fragments were sequenced that spanned the whole *ERG27* like gene.

gene, we used ERGP1down and ERGP2down which correspond to amino-acid sequences KRLTDVLVL and QASSSWFDC, respectively, in the sense direction of the formerly obtained gene fragment (Table 3). PCR amplifications in each case were performed in a 50 µl reaction volume containing 0.2 µg digested DNA, 1 µM each primer (one from the kit, the other one *ERG27* specific), 1 mM Mg^{2+}, 0.2 mM each dNTP and 0.1 unit ml^{-1} *Thermus thermophilus* polymerase mixed with a proof reading activity and *T. thermophilus* antibodies to allow hotstart PCR (Clontech). Amplification reactions used a touchdown procedure: seven cycles of 2 s at 94 °C and 3 min at 72 °C were followed by 32 cycles of 2 s at 94 °C and 3 min at 67 °C. Amplified fragments were visualized, purified through Qiagen columns and sequenced at the ESGS facility (Albertini et al., 1999). Sequences of primers are listed in Table 3. All the primers used in this study were synthesized by Genosys (UK).

cDNA cloning

Total RNA was extracted from ∼100 mg fresh mycelium of strain T4 (*transposa*, HydS) using Qiagen RNeasy Plant Mini Kit. DNA was digested with DNAse I and total purified RNA was submitted to RT–PCR (Clontech laboratories Inc., USA) following manufacturer's instructions. Specific *ERG27* primers: BEG, corresponding to the sequence MGLPPWETS, and END, corresponding to the sequence GRQRNAEPL (in the antisense direction of the gene) were designed to specifically amplify *ERG27* cDNA. PCR with primer pair BEG/END was performed in a 50 µl reaction volume containing 0.2 µg total cDNA. Reaction conditions and cycling were as described for the gene walking procedure. *ERG27* cDNA of the expected 1.6 kb size was visualized as a single band on 1% agarose gel, purified through a Qiagen column and directly sequenced = (Albertini et al., 1999).

Polymorphism analysis

The same primer pair BEG/END was used to amplify and sequence the whole *ERG27* gene from various *B. cinerea* field strains of *transposa* and*vacuma* types with different behaviours towards the 3-keto reductase inhibitor fenhexamid and the 14α-demethylase inhibitor prochloraz. Four fenhexamid sensitive strains of *S. sclerotium* were also included in this analysis.

Phylogenetic analysis

Homologues of *B. cinerea ERG27* sequence were identified by iterative PSI-BLAST search (Altschull et al., 1997) of the non-redundant protein database at NCBI. Position specific iterated BLAST uses an iterative search in which sequences found in one round of searching are used to build a score model for the next round of searching. Highly conserved positions receive high scores and weakly conserved positions receive scores near zero. The profile is used to perform subsequent BLAST searches and the results of each 'iteration' are used to refine the profile.

Protein sequences were aligned using ClustalW and their phylogeny analysed using the Fitch-Margoliash procedure of the Phylip software package (Felsenstein, 1989). The root of the phylogenetic tree was determined using fruitfly (*Drosophila melanogaster*) alcohol dehydrogenase (CAA66891); *Cochliobolus lunatus* 17β-HSD (AAD12052); *Streptomyces exfoliatus* 20β-HSD (S10707) and human 17β-HSD1 (AAB49519) as outgroups.

Results

Phenotypic characterization of strains

According to the *in vitro* responses of field strains of *B. cinerea* towards fenhexamid, four phenotypes were identified. First, the wild-type strains, HydS, which tolerated up to 0.08 mg l^{-1} fenhexamid, but were completely inhibited by 0.4 mg l^{-1}. These strains, either from *vacuma* or *transposa* types, had prochloraz EC_{50} values between 0.03 and 0.10 mg l^{-1} (Table 2). The second main phenotype, HydR1, tolerated up to 0.4 mg l^{-1} fenhexamid but was more sensitive to prochloraz having EC_{50} values below 0.02 mg l^{-1} (Table 2). Most of the HydR1 strains were of the *vacuma* type, but one of them contained *Flipper*, a single transposable element (Table 1). Comparison of both conidia length and mycelial growth rates between HydR1 and HydS strains also revealed differences between them, HydR1 strains having oversized conidia (Fournier et al., unpubl.) and a higher mycelial growth rate (Leroux et al., 2002a). Fertile crossing was recorded between HydR1 strains while we failed to obtain progeny in crosses between HydR1 and non-HydR1 strains. In vegetative pairings, there was a strong somatic incompatibility reaction between HydR1 strains and non-HydR1 strains, whereas all the

tested HydR1 strains were compatible (Leroux et al., 2002a).

The other fenhexamid-resistant strains: HydR2 and HydR3, which were of the *transposa* type, did not exhibit increased sensitivity towards sterol biosynthesis inhibitors. They could be divided into two groups according to the *in vitro* effect of fenhexamid towards germ-tube elongation (Leroux et al., 2002b). HydR2 strains, like the HydR1 ones, tolerated up to 0.4 mg l^{-1} fenhexamid, whereas HydR3 strains were more resistant: germ-tube elongation in these strains occurred in the presence of up to 4 mg l^{-1} fenhexamid (Table 2). Resistance to dicarboximides (ImiR), whose putative target could be an histidine kinase, has not previously been recorded in fenhexamid-resistant strains (Table 1). On the other hand, resistance to antimicrotubule fungicides, such as the benzimidazoles, was frequently encountered in strains of both *vacuma* and *transposa* types irrespective of their Hyd phenotypes (Table 1). The AniR3 strain (154) was only weakly resistant to fenhexamid, dicarboximides, DMIs and anilinopyrimidine (pyrimethanil) as a result of a multidrug resistance phenomenon (Chapeland et al., 1999).

The four *S. sclerotium* strains reported in this study were found to be as sensitive to fenhexamid as the *B. cinerea* HydS strains (Table 1).

Table 2. Response of the various phenotypes of *B. cinerea* towards two fungicides: fenhexamid and prochloraz

Phenotype (target)	Fenhexamid[a]		Prochloraz[b]
	0.4	4	0.02
HydS	−	−	+
HydR1	+	−	−
HydR2	+	−	+
HydR3	+	+	+

[a]For fenhexamid, effects shown were on elongation of germ-tubes produced by conidia. The discriminating concentrations are given in mg l^{-1}. −: no germination or presence of short germ-tubes; +: presence of long germ-tubes.

[b]Prochoraz effects were measured on mycelial growth. The extreme concentration given here (in mg l^{-1}) allowed us to distinguish the HydR1 type which EC_{50} value is below 0.02 mg l^{-1} from HydS, HydR2 and HydR3 types which EC_{50} are higher than 0.02 mg l^{-1}. −: no mycelial growth or less than 50% of the control; +: more than 50% of the control growth.

Cloning the ERG27 *gene*

Genome walking

Genome walking on *B. cinerea* DNA was conducted to obtain complete genomic *ERG27* sequences by performing PCR amplifications both upstream and downstream of the sequenced EST fragment. Amplifications of *transposa* T4 strain *Dra*I-digested DNA ligated to synthetic adaptors by using primer pairs ERGPup/AP1 and ERGPup/AP2 successively (Table 3), yielded a main PCR product of roughly 1.2 kb. This fragment encompassed a partial ORF of 1020 bp including the first 141 bp of the EST fragment as well as a unique 79 bp intron. Amplifications performed with *transposa* T4 strain *Dra*I-digested DNA ligated to the same synthetic adaptors using primers pairs ERGP1-down/AP1 and ERGP2-down/AP2 yielded a single PCR product of ∼1 kb. This fragment, including the last 372 bp of the EST fragment, contained a partial ORF of 897 bp terminated by a TGA codon. As sequenced fragments overlapped with the EST fragment, we could unambiguously define three contigs. The 1686 bp long nucleotide sequence of the *B. cinerea ERG27* like gene resulted therefore from joining overlapping sequences of the upstream 1.2 kb, the 537 bp EST fragment and the downstream 1 kb fragments (Figure 1A–C).

ERG27 *cDNA*

The *B. cinerea ERG27* like gene was interrupted by a single putative intron at nucleotide position 247–302.

Table 3. Primers designed for this study

Primer's name	S or R*	Nucleotidic sequence	Amino-acid sequence	Relative position (codons numbers)
ERGPup	R	5′-AATTCCAGGTTGAACCACGCATTTTCG3′	YVVQPGIF	393–386
ERGP1down	S	5′-CAAACGTCTTACAGATGTTCTCGTCCTTTC3′	KRLTDVLVL	337–345
ERGP2down	S	5′-CCAAGCCTCCTCTTCATGGTTCGATTGTTC3′	QASSSWFDC	354–362
BEG	S	5′-TGGGATTACCACCATGGGAGACAAGTG3′	MGLPPWETS	1–8
END	R	5′-CAATGGTTCCGCATTTCTTTGCCTCCC3′	GRQRNAEPL	501–493

*S for sense, R for reverse.

A comparison of PCR products, obtained after using primers pair BEG/END on genomic DNA, and cDNA revealed that the intron really excised was in fact longer than the conceptual one. The remainder of the cDNA coding sequence was identical to that of the *ERG27* genomic DNA, thus further confirming the *ERG27* gene was not a pseudogene. The complete *ERG27* sequence of *B. cinerea* T4 strain can be found in GenBank under the accession number AY220532.

Sequences analysis

The inferred 535 amino-acid protein encoded by the putative *ERG27* gene from the *B. cinerea* T4 strain (*transposa*, HydS) possesses features common to reductases, including an active catalytic site made up of a tyrosine followed by a lysine four residues downstream (YXXXK) and a N terminal NADP(H) binding site requiring three glycine residues in a characteristic GXXXGXG pattern (reviewed by Jörnvall et al., 1995; Peltoketo et al., 1999). When compared to known Erg27p sequences available in the databases, *S. cerevisiae* and *Candida albicans* 3-keto reductases (GenBank accession numbers: NP_013201 and AY140908, respectively) the *B. cinerea* Erg27p like protein displayed up to 30% identity. The *B. cinerea* protein is longer than that of the yeasts, having 535 amino-acid residues instead of 347 (346 in the case of *C. albicans*). Removing gapped positions increased homology levels up to 35% identity. Enzymatic assays had shown that C4-demethylation location is microsomal both in yeast and in *B. cinerea* (Gachotte et al., 1999; Debieu et al., 2001). Although the Erg27p enzyme from yeast had no transmembrane domain and is anchored into the microsomal membrane by protein–protein interactions (Mo et al., 2002), the *B. cinerea* Erg27p like protein had a predicted transmembrane helix between amino-acid positions 386 and 408 (http:/www.cbs.dtu.dk/service/TMHMM).

A *Schizosaccharomyces pombe* hypothetical protein (GenBank accession number: BAA13878) having 29% sequence identity with the *S. cerevisiae* enzyme, displayed also 30% identity with the *B. cinerea* Erg27p like protein. A 475 amino-acid *Neurospora crassa* hypothetical protein (GenBank accession number: EAA29563) that was obtained after conceptual identification of ORFs of the whole genome was also found to have significant homology with the *B. cinerea* Erg27p like protein (39% identity).

A BLAST search identified 17β-hydroxysteroid dehydrogenase type 7 (17β-HSD7) enzymes from mammals as close homologues to the *B. cinerea* Erg27p like protein. For example, the *B. cinerea* Erg27p like protein showed 25% identity with the human 17β-HSD7 enzyme (GenBank accession number: CAC88111), and up to 28% identity with the mouse one (GenBank accession number: NP_034606). Removal of gapped positions resulted in increased homology levels. Besides amino-acid sequence conservation, including a typical NAGI motif whose significance is unknown, the unique intron of the *B. cinerea* *ERG27* like gene is at the same position as the first intron of the mammalian 17β-HSD7 gene (Figure 2).

In contrast, the different types of human 17β-HSD enzymes which are involved in steroid hormone metabolism, especially conversion of inactive estrone to its biologically active hydroxy form estradiol, do not display more than 15% identity between each other. For instance, human 17β-HSD types 1 and 7 share 14% identity, types 7 and 8 share 14% identity whereas sequence comparison between 17β-HSD7 and 17β-HSD type 4 which is longer than other types of 17β-HSD (736 amino-acid long) shows these enzymes share only 9% sequence identity.

The only known HSD from fungal origin is the soluble 17β-HSD of *C. lunatus* that was purified and characterized through its reductase properties on mammalian steroid hormones (Lanisnik Rizner et al., 1996). However, after cloning and sequencing, this enzyme was not found to be homologous to mammalian 17β-HSD7 (15% identity) nor to *S. cerevisiae* Erg27p (14% identity). *B. cinerea* Erg27p like protein did not display more than 11% identity with the *C. lunatus* enzyme. The *C. lunatus* enzyme is closer to the mammalian 17β-HSD8 one (25% identity) which is not homologous to 17β-HSD7 (14% identity) nor to *B. cinerea* Erg27p like protein (12% identity).

Iterative PSI-BLAST search (Altschul et al., 1997) of the non-redundant protein database at NCBI was performed to increase BLAST sensitivity. Results confirmed sequence homology between the *B. cinerea* Erg27p like protein, mammalian 17β-HSD7 enzymes and yeast 3-keto reductases. Alignment of protein sequences followed by phylogeny analysis using the Fitch-Margoliash procedure of the Phylip software package (Felsenstein, 1989) also showed the relationship between mammalian 17β-HSD, yeast 3-keto reductases and the *B. cinerea* Erg27p like protein which

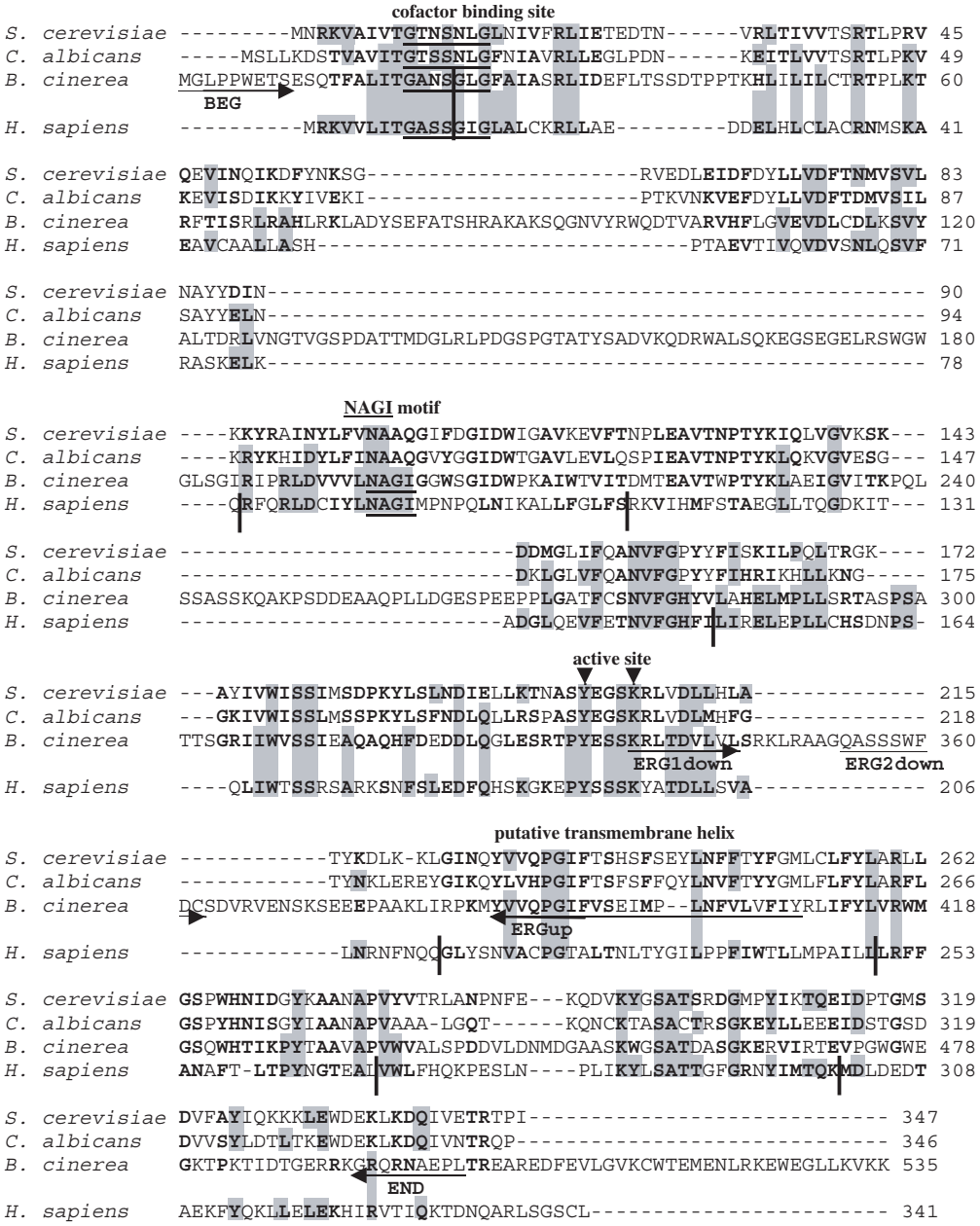

Figure 2. ClustalW alignment of the amino-acid sequences encoded by the *ERG27* genes from *S. cerevisiae*, *C. albicans*, the *B. cinerea* putative *ERG27* gene and the human 17β-HSD7 gene (http://www2.ebi.ac.uk/clustalw). Shaded areas indicate regions of sequence identity. Similar residues when found at least in the human enzyme and in one of other sequences or alternatively in the three fungal sequences are shown in boldface. Human exons boundaries are indicated by vertical bars, note that the unique *B. cinerea* exon boundary is located at the same position as the first human one (yeasts *ERG27* genes are devoided of intron). NADPH cofactor binding sites, NAGI motif and putative transmembrane domain are underlined. The two residues involved in active site, i.e., Y and K (consensus sequence YXXXK) are indicated by arrowsheads. Primers used in this study are namely indicated by horizontal arrows under the *B. cinerea* sequence.

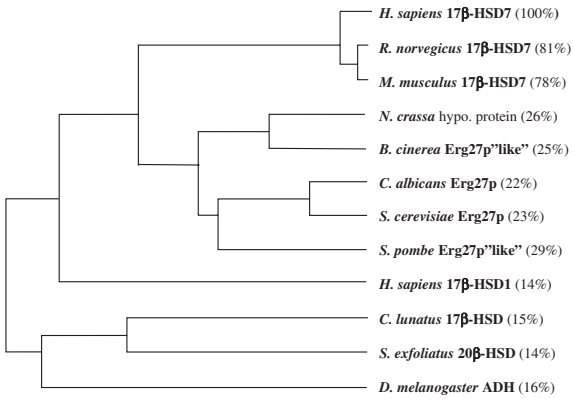

Figure 3. Phylogenetic tree of *B. cinerea* Erg27p like protein, mammalian 17β-HSD7 and yeasts Erg27p. The relationship among these proteins was determined by a Fitch-Margoliash analysis with parsimony bootstrap replications using the Phylip software package (Felseinstein, 1989). GenBank accession numbers of representative proteins used for the analysis: human 17β-HSD7 (*Homo sapiens*): CAC88111; mouse 17β-HSD7 (*Mus musculus*): NP034606; rat 17β-HSD7 (*Rattus norvegicus*): NP058931; *B. cinerea* Erg27p like: AY220532; *S. cerevisiae* Erg27p: NP_013201; *S. pombe* putative Erg27p: BAA13878; *C. albicans* Erg27p: AY140908; *N. crassa* hypothetical protein: EAA29563. Fruitfly alcohol dehydrogenase (*D. melanogaster*): CAA66891; *C. lunatus* 17β-HSD: AAD12052; *S. exfoliatus* 20β-HSD: S10707; human 17β-HSD1: AAB49519 were used as outgroup.

form a highly divergent group among a larger family of short-chain alcohol dehydrogenase (Figure 3).

Erg27p like protein variability

To assess if we could find a relationship between differential sensitivity to fenhexamid and mutations at the Erg27p like protein level, primers pair BEG/END was used to amplify and sequence the *ERG27* gene from 11 *B. cinerea* strains which had been characterized with respect to fungicide resistance and the presence of transposable elements (Table 1). After conceptual translation, the 11 sequences clustered in two main groups, HydR1 and non-HydR1 ones. There was only one allele for the HydR1 strains and three for the non-HydR1 ones. Sequence comparison of the four different alleles between position 15 and 515 of the 535 amino-acid gene product revealed 15 polymorphic positions (Figure 4). Among these polymorphic expressed positions, 12 clearly enable us to distinguish HydR1 from non-HydR1 sequences (Figure 4). These 12 mutations were as follows: a valine as in the human 17β-HSD7 sequence (instead of an asparagine) at position 93, an asparagine (instead of an aspartic acid) at 146, a valine as in the *S. cerevisiae* Erg27p sequence (for an isoleucine) at 211, a leucine (for an isoleucine) at 215, a threonine (for a methionine) at 218, an alanine (for a valine) at 234, a valine (for an isoleucine) at 235, a glycine (for an aspartic acid) at 261, a threonine (for a serine) at 264, a leucine (for a proline) at 269, a threonine (for an alanine) at 285 and a lysine (for a glutamine) at 354 of the protein. They were all present in the three HydR1 strains we analysed. Three other mutations were found in non-HydR1 strains: a serine (instead of a proline) at 238, an isoleucine (for a phenylalanine) at 412 and a threonine for an arginine at position 496. Among these, the 246 mutation (S for P) was observed both in the two HydR3 strains, in a HydS one (L) as well in a multidrug resistant strain (Ani R3, 154), but the two latest (I for R at 412 and T for R at 496) which substituted the same residues as in the human 17β-HSD7 sequence, allowed us to distinguish HydR3 strains from other non-HydR1 ones (Figure 4).

We did not find any mutations in the protein sequences from HydR2 strains or from the multidrug resistant AniR3 strain that were thus undistinguishable from the T4 HydS one (data not shown).

At the nucleotide sequence level, the 79 bp intron was characterized by four dimorphic regions (data not shown) which allowed the three HydR1 strains to be distinguished from the non-HydR1 ones (all having the same intron sequence whatever their Hyd phenotype: HydS, HydR2, HydR3 or AniR3).

The four *S. sclerotium* Erg27p like protein sequences analysed were all identical, showing 82 differences with the T4 *B. cinerea* Erg27p like protein corresponding to 78% identity due to their close phylogenetic position (data not shown).

Discussion

The *ERG27* like *B. cinerea* gene which was cloned, was expressed and is likely to be a reductase gene. The inferred 535 amino-acid protein possesses the two common features of most reductases: the active catalytic site whith a consensus sequence of YXXXK and the characteristic GXXXGXG pattern of the N terminal NADP(H) binding site. Moreover, the Erg27p like protein also has the NAGI motif which is encountered in mammalian 17β-HSD7.

Reductases are poorly conserved proteins. The primary structures of HSD enzymes are not very

Figure 4. Erg27p like protein polymorphism among the different *B. cinerea* Hyd phenotypes (between amino-acid positions 70 and 500). Mutations in the *B. cinerea* Erg27p like protein sequences are shown in shaded boxes and the corresponding phenotypes are indicated in the left column. The NAGI motif (also found in mammalian 17β-HSD7) and the reductase active site are shown in boxes, the putative transmembrane helix is delimited by arrowheads.

similar. For instance, the different types of human 17β-HSD enzymes do not display more than 15% identity between each other. This poor homology level is likely to indicate either distant duplication and early divergence or a convergent evolution phenomenon from different ancestral proteins as for 17β-HSD types 3 and 5 (Baker, 2001). Therefore, the observed homology between *B. cinerea* putative Erg27p enzyme and mammalian 17β-HSD7 is noteworthy. That could suggest, despite the phylogenetic distance between mammals and fungi, a common ancestor. High sequence conservation between these proteins might be associated with a common function.

Breitling and colleagues have presented evidence that 17β-HSD7 could be an ancient 3-keto reductase of cholesterogenesis (Breitling et al., 2001a). Based on *in silico* Northern blot experiments and phylogenetic analysis, they suggested that 17β-HSD7 might have two distinct roles: a role in estrone conversion to estradiol and an additional one. As they unexpectedly found that the liver, which is the location of estradiol inactivation, is also the predominant site of expression of human 17β-HSD7, they suggested that, in liver, 17β-HSD7 might convert a substrate that differs from estrone. Sequence homology between yeast Erg27p and 17β-HSD7 led them to propose that 17β-HSD7 was involved in cholesterol biosynthesis as a 3-keto reductase enzyme (Breitling et al., 2001a; Husen et al., 2003). This hypothesis is particularly interesting, since up to now, attempts to solubilize a mammalian 3-keto reductase of cholesterogenesis and to demonstrate its non-identity to other hepatic 3-keto reductases involved in steroid hormone metabolism were unsuccessful (Gaylor, 2002).

However, homology between *C. lunatus* 17β-HSD and mammalian 17β-HSD8 was not helpful in assigning a physiological role to the fungal enzyme. It is not clear whether *C. lunatus* 17β-HSD was related to steroid signalling in connection with the endogenous biosynthesis of androgens and androgen-binding proteins, to biosynthesis of melanin or mycotoxins, or to detoxification mechanisms in the fungus (Lanisnik Rizner et al., 2001). This uncertainty about the role of the 17β-HSD in fungi is nevertheless interesting as it could suggest the emergence of a new function during the evolution of this particular gene in mammals or

alternatively the possibility that 17β-HSD participates in different biochemical processes in the same way as for the 17β-HSD type 4 enzyme whose expression does not correlate with estradiol dehydrogenase activity in several tissue types (Breitling et al., 2001b). In yeast, Erg27p, besides its 3-keto reductase activity, appears to act as a chaperon of Erg7p and could therefore be considered as a multifunctional protein (Mo et al., 2003).

On the other hand, variability analysis of the *B. cinerea* Erg27p like protein gave additional support to our previous work (Albertini et al., 2002). Using systematic sequencing of the *ERG27* like gene, we found that HydR1 strains constitute an homogeneous group that could be unambiguously distinguished from non-HydR1 strains. Twelve expressed mutations, that could be involved in the natural resistance of these strains to fenhexamid, as well as the sequence of the unique intron, are the characteristic features of HydR1 *ERG27* like gene. Therefore these results confirmed previous results obtained with the *CYP51* gene (Albertini et al., 2002), the *BC-hch* gene (Fournier et al., 2003) as well as with four other nuclear loci (Fournier et al., in press) which indicated that HydR1 and non-HydR1 strains are in fact two distinct species.

Furthermore, Erg27p like protein could give us an additional marker for the discrimination of HydR3 strains among non-HydR1 strains. Two mutations that substitute residues usually encountered in mammalian 17β-HSD7 have been found in HydR3 strains and could play a role in acquired resistance to fenhexamid. However, HydR3 strains are rare and more experiments are needed to conclude.

As preliminary transcription/translation experiments have shown that the putative *B. cinerea ERG27* gene is efficiently translated *in vitro* in the presence of microsomes, our present goal is to set up a biochemical assay in order to check whether the *B. cinerea* enzyme is a 3-keto-reductase activity or alternatively to find out its biochemical function(s).

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