Biodegradation of Phenol by Antarctic Strains of *Aspergillus fumigatus*

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Taxonomic identification of three newly isolated Antarctic fungal strains by their 18S rDNA sequences revealed their affiliation with *Aspergillus fumigatus*. Phenol (0.5 g/l) as the sole carbon source was completely degraded by all strains within less than two weeks. Intracellular activities of three key enzymes involved in the phenol catabolism were determined. Activities of phenol hydroxylase (EC 1.14.13.7), hydroquinone hydroxylase (EC 1.14.13.x), and catechol 1,2-dioxygenase (EC 1.13.11.1) varied significantly between strains. The rates of phenol degradation in the three strains correlated best with the activity of catechol 1,2-dioxygenase.

Six pairs of oligonucleotide primers were designed on the basis of the *Aspergillus fumigatus* Af293 genome sequence (NCBI Acc. No. XM_743491.1) and used to amplify phenol hydroxylase-related gene sequences. DNA sequences of about 1200 bp were amplified from all three strains and found to have a high degree of sequence identity with the corresponding gene of *Aspergillus fumigatus* Af293.

Key words: Phenol, *Aspergillus fumigatus*, Phenol Hydroxylase Gene

Introduction

The capability of Antarctic microorganisms, including filamentous fungi, to exist in some of the most severe climatic conditions known on earth, such as extremely low temperatures and poor availability of nutrients, is the cause of increasing interest in their metabolism. The study of species diversity characteristics of this region and the organisms' metabolic capabilities can provide new solutions for industry and environment. Many strains isolated from this area which is characterized by very low temperatures are psychrotrophs (growth temperatures from 15 to 20 °C), and only a few of them are true psychrophiles (Margesin *et al*., 2003, 2005; Tosi *et al*., 2010).

Phenol and its various derivatives, as well as many other aromatic compounds, are known as some of the most hazardous pollutants (Smith *et al*., 1988; Sikkema *et al*., 1995). Many phenol-contaminated environments are characterized by low temperatures. With this in mind, the microorganisms adapted to growth in these conditions might play an important role in the bioremediation of such polluted habitats (Margesin *et al*., 2005). Most of the published investigations on the degradation of phenol were performed with strains of bacteria and also of some yeasts (Pakula *et al*., 1999; Alexieva *et al*., 2008; Nair *et al*., 2008; Chakraborty *et al*., 2010). Some strains of hyphal fungi, such as *Fusarium*, *Graphium*, and *Aspergillus*, have been cited for their potential for phenol degradation (Anselmo *et al*., 1985; Santos and Linardi, 2004; Krastanov *et al*., 2009; Yemendzhiev *et al*., 2009). Many moulds have been isolated from soil probes taken in Antarctica, and many of them demonstrated good tolerance to the presence of toxic phenolic compounds in their culture medium (Aislabie *et al*., 2006; De Domenico *et al*., 2004; Alexieva *et al*., 2011).

The efficient degradation and utilization of toxic environmental pollutants, such as phenol, depends on the availability and activity of some key enzymes in the microbial cells. The first step in the aerobic metabolism of phenol is its *ortho*-hydroxylation to catechol by phenol hydroxylase. Catechol is a central intermediate in the degradation pathways of various aromatic compounds; it is metabolized by different strains via either the *ortho*- or *meta*-cleavage pathway (Neujahr and Varga, 1970; Reardon *et al*., 2000; Stoilova *et al*., 2006; Pradhan and Ingle, 2007). The *ortho*-mechanism has been

reported for all investigated mould strains. There are some reports on a different mode of ring cleavage in fungi, acting in parallel to the hydroxylation to catechol (Fig. 1). In this catabolic pathway, phenol is first converted by *para*-hydroxylation to hydroquinone and then further hydroxylated by hydroquinone hydroxylase to 1,2,4-trihydroxybenzene. Both catechol 1,2-dioxygenase and 1,2,4-trihydroxybenzene dioxygenase catalize *ortho*-ring-cleavage (Jones *et al*., 1995; Claussen and Schmidt, 1998; Eppink *et al*., 2000).

Polymerase chain reaction (PCR) and DNA sequence analysis have been successfully applied in the study of genes involved in the catabolism of aromatic compounds (Okuta *et al*., 1998; Kahng and Oh, 2005; Manasiev *et al*., 2008). For example, the gene Cvmp encoding phenol mono-oxygenase in *Chromobacterium violaceum* was identified by its homology (74% similarity and 59% identity) to the gene encoding phenol hydroxylase in the bacterium *Ralstonia eutropha* (Perpetuo *et al*., 2009). These approaches undoubtedly expand our knowledge of the biodiversity of microorganisms and of their potential to degrade aromatic compounds (Futamata *et al*., 2001; Todorova, 2010; Tautz *et al*., 2010).

The aim of this study was to specify the taxonomic affiliation of three newly isolated Antarctic *Aspergillus* strains, and to examine their biochemical and genetic potential utilizing phenol as a carbon source. We investigated the activities of the intracellular enzymes catalyzing some of the initial steps of phenol catabolism and identified and sequenced the gene encoding phenol hydroxylase in three of the strains.

Material and Methods

Microorganisms

Earlier, a multitude of filamentous fungi had been isolated from soil samples collected on Liv-

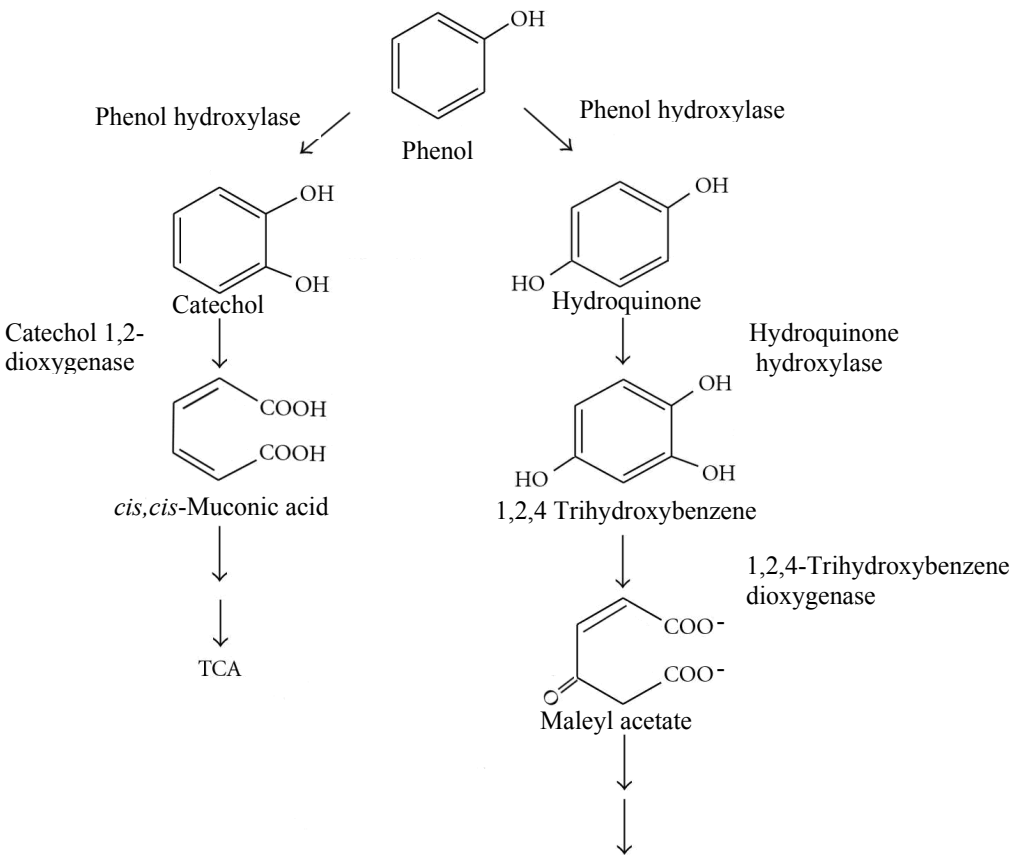

Fig. 1. The *ortho*- and *para*-hydroxylation pathways of phenol degradation in fungi.

ingston Island, Antarctica by the Bulgarian expedition conducted in January and February 2007 (Kostadinova *et al*., 2009). Fungi were isolated from the samples on Cooke Rose Bengal agar medium (5.0 g/l soy peptone, 10.0 g/l dextrose, 1.0 g/l NaH_2PO_4, 0.5 g/l $MgSO_4$, 20.0 g/l agar, 0.035 g/l Rose Bengal, and 0.2 g/l chloramphenicol) (Sigma-Aldrich, Taufkirchen, Germany) by soil dilution and soil sprinkle techniques. Taxonomic identification of 16 isolates was performed based on morpho-dimensional parameters following the available identification keys for the different genera (Tosi *et al*., 2002).

Microbial cultivation

Fungi growing on the agar medium were subcultured by transfer of spores to fresh beer agar medium. Six ml spore suspension of a 7- to 8-day-old culture in 0.1% Triton X-100 medium were used as inoculum for cultivation in Czapek-Dox medium (2 g/l $NaNO_3$, 1 g/l KH_2PO_4, 0.5 g/l KCl, 0.5 g/l $MgSO_4 \cdot 7\ H_2O$, 0.01 g/l $FeSO_4 \cdot 7\ H_2O$) supplemented with 1% glucose at pH 5.5. To ensure equal amounts of biomass in the inocula, fungal pellets were aseptically filtered and (70 ± 5) mg mycelium (fresh weight) were used for inoculation in 50 ml Czapek-Dox medium, containing phenol in appropriate concentrations as sole carbon source, in 300-ml flasks. The flasks were agitated on a New Brunswick (Enfield, CT, USA) rotary shaker (240 rpm) at 23 °C. Samples were taken at 24-h intervals and centrifuged at 2350 x *g* for 20 min for recovery of the mycelia (Yemendzhiev *et al*., 2009).

Cell-free extracts were obtained by mechanical grinding of the mycelia in 2 ml buffer containing 0.06 M each of KH_2PO_4 and Na_2HPO_4 (pH 7.6). The obtained lysates were clarified by centrifugation (5 min, 4700 x *g*, 4 °C).

Analytical methods

Phenol hydroxylase (EC 1.14.13.7) activity was determined spectrophotometrically at 340 nm by following the NADPH oxidation in the presence of phenol as described by Neujahr and Gaal (1973). Catechol 1,2-dioxygenase (EC 1.13.11.1) activity was determined spectrophotometrically by the increase in the absorption at 260 nm, due to the accumulation of *cis,cis*-muconic acid (Varga and Neujahr, 1970). Hydroquinone hydroxylase (EC 1.14.13.x) activity was followed by the oxidation of NADPH at 340 nm. The assay mixture (1.0 ml) contained 42 mM KH_2PO_4 (pH 7.1), 0.15 mM NADPH, 1 mM hydroquinone, and cell extract (Jones *et al*., 1995).

All enzyme activities were measured at 25 °C and expressed in units (U)/mg protein. One unit of phenol hydroxylase or hydroquinone hydroxylase activity is defined as the amount of enzyme that oxidizes 1 μmol of NADPH within 1 min. One unit of catechol 1,2-dioxygenase activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol *cis,cis*-muconic acid per min.

The phenol concentration was determined by a residual colorimetric method with the basic reagent 3,4-dimethyl amino antipyrine (Hristov, 1997). The concentration of proteins in the cell-free lysate was determined by the method of Lowry *et al*. (1951) with bovine serum albumin (Sigma-Aldrich) as standard.

DNA isolation procedure

Fifteen ml YEPD medium (10.0 g/l yeast extract, 20.0 g/l peptone, 10 g/l glucose) were inoculated with a single fungal colony and incubated in 100-ml Erlenmeyer flasks for 48 h on a rotary shaker at 180–200 rpm and 23 °C. After centrifugation at 1500 x *g* for 15 min the resulting pellet was washed with 5 ml 0.98% saline solution and centrifuged as before. The pellet was ground in a mortar with quartz sand for about 10 min. Two ml 1xTE buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA) were added to the cell lysates and the homogenates dispensed in Eppendorf tubes (500 μl/tube). The samples were centrifuged at 13850 x *g* and the resulting supernatant was used for DNA isolation according to Maniatis *et al*. (1982). The DNA was purified via GFX columns (GE Healthcare, Little Chalfont, Buckinghamshire, UK), and its quality and quantity checked by measurement of the UV absorption.

PCR conditions and DNA sequencing

The list of the used primers is shown in Table I.

The amplification was performed on an Eppendorf Mastercycler personal thermocycler (Eppendorf AG, Hamburg, Germany) using PuReTaq™ Ready-To-Go™ PCR beads (Amersham Biosciences, Piscataway, NJ, USA). The final concentration of primers in the reaction mixture was 0.4 pmol/μl. The concentration of the DNA matrix was 50 ng/25 μl (final volume of the reaction mixture).

Table I. List of primers and PCR conditions used in the experiments.

Primer	Sequence (5' → 3')	Source
PFf PFr	AGGGATGTATTTATTAGATAAAAAATCAA CGCAGTAGT-TAGTCTTCAGTAAATC (PCR conditions: initial step, 95 °C, 5 min; 35 cycles amplification, 95 °C, 30 s; 58 °C, 30 s; 72 °C, 45 s; extension step, 72 °C, 7 min)	Universal primers for fungal 18S rDNA amplification (Jaeger *et al.*, 2000)
pAF1f pAF2r	GTCTTGAGGTCTCAAGGATGAATT TAGGTGAAATG-GTTGGCAACTCTC GTATCTCGAGAAACGGCGTGGAGA (PCR conditions: initial step, 95 °C, 5 min; 35 cycles amplification, 95 °C, 30 s; 54 °C, 30 s; 72 °C, 45 s; extension step, 72 °C, 1 min)	Primers for detection of phenol hydroxylase-coding genes designed in this study on the basis of *Aspergillus fumigatus* Af293 sequence (NCBI Acc. No. XM_743491.1)
pPHF3f pPHF4r pPHF5f pPHF6r	GCGACTGGTGGACAATCTAC CGTAGTTGCGCAGCTGCT-CATA TATGAGCAGCTGCGCAACTACG TCAGCGCGTGAA-GATGGGATG (PCR conditions: initial step, 95 °C, 5 min; 35 cycles amplification, 95 °C, 30 s; 52 °C, 30 s; 72 °C, 1 min; extension step, 72 °C, 5 min)	As above

The obtained PCR products were purified by the GFX™ PCR DNA and gel band purification kit (GE Healthcare). The reaction mixture for DNA sequencing contained 2 μl of purified PCR product (2 ng/μl), 1.3 μl Big Dye® terminator kit v. 3.1, 2 μl 5x Seq buffer, and 0.35 μl primers (10 pmol/μl). The volume was brought to 10 μl with distilled water. The amplified fragments were sequenced on an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Comparative sequence analysis

The raw data obtained from the sequencing were checked for errors by the program Sequence Scanner V1.0 (Applied Biosystems). DNA sequences were formatted in a form suitable for comparison by the BLAST analysis database of the National Center for Biotechnology Information (NCBI). The corresponding protein sequences were established from the DNA sequence using DNASTAR Inc. (Madison, WI, USA) software. The pair and multiple sequence alignment were performed using ClustalW 2.

Agarose gel electrophoresis

DNA was resolved on a 0.7 or 1.5% agarose gel with TBE buffer (10.8 g/l Tris base, 5.5 g/l boric acid, 20 ml of 0.05 M EDTA, pH 8.0) by electrophoresis, stained with ethidium bromide, and visualized by UV irradiation at 254 nm. The DNA fragment sizes were estimated by comparison with a 3 kb 50- to 3000-bp DNA ladder (Sigma-Aldrich) and an 1.5 kb 100-bp DNA ladder (Thermo Fisher Scientific, Waltham, MA, USA).

Results and Discussion

Taxonomic identification

The panfungal primer pair PFf and PFr (Table I) was used to amplify 18S rDNA sequences to determine the taxonomic affiliation of three Antarctic fungal strains. The comparison of the obtained 18S rDNA sequences with those of reference organisms was done in the gene sequence database of the NCBI. The validation of the genotypic vs. the phenotypic analyses indicated that the investigated strains are closely related to the species *Aspergillus fumigatus* (Tosi *et al.*, 2010). The obtained nucleotide sequences were registered in the NCBI database under the following accession numbers: *A. fumigatus* AMA1102 (HM231098.1); *A. fumigatus* AL8 (JN206689.1); *A. fumigatus* AL9 (JQ639072.1) (Fig. 2).

Biodegradation experiments

Mould strains have been reported to degrade phenol with different degrees of efficiency. For example, a strain of *Graphium* sp. degraded 0.75 g/l phenol within 168 h (Santos and Linardi, 2004), *Aspergillus* sp. LEBM2 degraded 0.5 g/l phenol within 144 h (Passos *et al.*, 2010), and *Aspergillus awamori* NRRL 3112 degraded 0.5 g/l phenol within 72 h (Yemendzhiev *et al.*, 2009).

All *Aspergillus* strains studied in this work had earlier been found to exhibit a good phenol tolerance, being able to grow in a medium containing up to 0.7 g/l phenol (Alexieva *et al.*, 2011). In a medium containing 0.5 g/l phenol as a sole carbon and energy source, they completely degraded the compound within 9–11 days with similar kinetics (Fig. 3). In

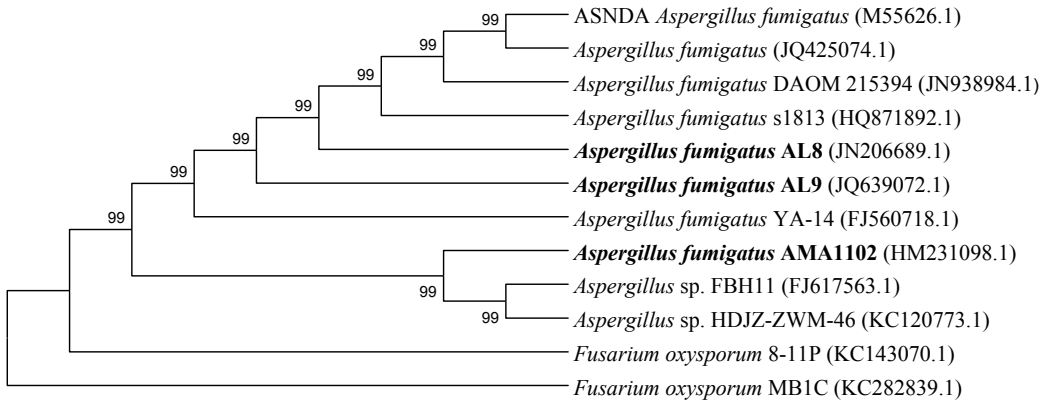

Fig. 2. Cladogram created from 18S rDNA sequences of *Aspergillus* strains AL8, AL9, AMA1102 and sequences of the closest phylogenetic neighbours obtained from NCBI data. Two *Fusarium oxysporum* strains were used as an out-group. The tree was constructed using MEGA 4.1 software.

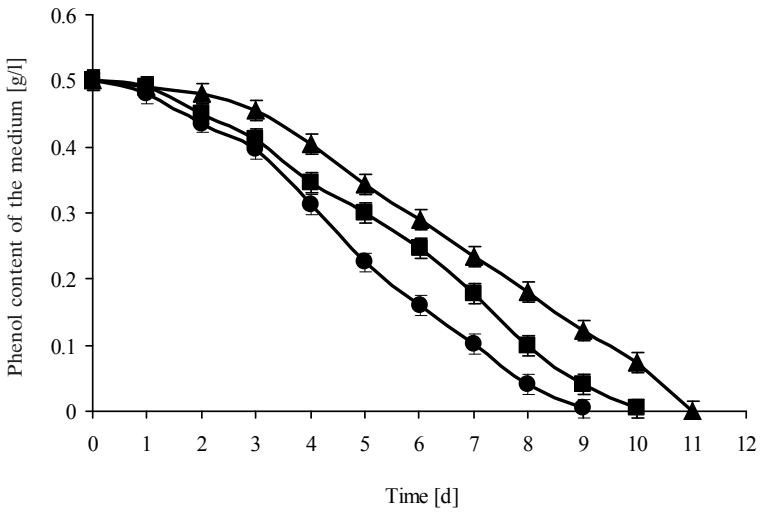

Fig. 3. Time course of phenol degradation by *Aspergillus fumigatus* strains grown in mineral medium with 0.5 g/l phenol as the sole carbon source: ● AL8; ■ AMA1102; ▲ AL9. Mean values ± SD of three replicates are shown.

the absence of the fungi, the phenol concentration was stable. In the presence of phenol as well as a low glucose content (1%), the fungi formed mycelial pellets, while in the presence of more than 2% glucose no pellet formation occurred.

At 0.3 g/l phenol concentration, degradation was complete within 72 h, while at 0.6 g/l only 50–90% had been degraded after 17 days, depending on the strain (data not shown). Similar observations on phenol degradation as a function of its concentration have been reported by others (Sikkema *et al.*, 1995; Jiang *et al.*, 2005; Kumar *et al.*, 2005).

Enzyme activities

Activities of the enzymes phenol hydroxylase, catalyzing the *ortho*- or the *para*-oxidation of phenol yielding catechol or hydroquinone (Leitão, 2009), respectively, and hydroquinone hydroxylase, catalyzing the hydroxylation of hydroquinone to 1,2,4-trihydroxybenzene, were determined in crude extracts from the strains growing in the presence of 0.3 g/l phenol (Table II) in either the exponential growth phase (after 29 h) or at the onset of the stationary growth phase (after 72 h). The enzyme catechol 1,2-dioxygenase,

Table II. Intracellular activities of phenol hydroxylase, catechol 1,2-dioxygenase, and hydroquinone hydroxylase in mycelia of *Aspergillus* strains, cultivated in the presence of 0.3 g/l phenol, during early (29 h) and late (72 h) exponential growth phase.

Enzyme [U/mg protein]	Strain AMA1102		Strain AL8		Strain AL9	
	29 h	72 h	29 h	72 h	29 h	72 h
Phenol hydroxylase	0.093 ± 0.004	0.089 ± 0.003	0.189 ± 0.009	0.243 ± 0.006	0.098 ± 0.003	0.075 ± 0.003
Hydroquinone hydroxylase	0.080 ± 0.003	0.1 ± 0.008	0.005 ± 0.001	0.11 ± 0.002	0.109 ± 0.005	0.04 ± 0.002
Catechol 1,2-dioxygenase	0.956 ± 0.019	0.213 ± 0.013	1.116 ± 0.013	0.496 ± 0.014	0.432 ± 0.010	0.33 ± 0.009

Mean values ± SD of three replicates are shown.

catalyzing the second step in the *ortho*-pathway, was analysed in the same conditions. The activities of these enzymes varied significantly between the strains at both time points. A decrease of the activities of phenol hydroxylase with incubation time was observed in strains AMA1102 and AL9, but not in strain AL8. The activity of hydroquinone hydroxylase of strain AL9 decreased from 29 h to 72 h, while it increased slightly towards 72 h in the other two strains. The strains AMA1102 and AL9 exhibited comparable activities for the two hydroxylases. The best-growing strain AL8 possessed a 37.8 times higher phenol hydroxylase activity than hydroquinone hydroxylase activity in the log phase.

The obtained results showed the ability of the strains AMA1102 and AL9 to degrade phenol simultaneously by the two different pathways. The first one is the classic *ortho*-cleavage pathway going through catechol, in the second phenol is first converted by *para*-hydroxylation to hydroquinone and then further hydroxylated by hydroquinone hydroxylase to 1,2,4-trihydroxybenzene. Both catechol 1,2-dioxygenase and 1,2,4-trihydroxybenzene dioxygenase catalyze *ortho*-ring-cleavage. Similar results were reported in other publications related to fungal phenol degradation (Jones *et al*., 1995; Nakamura *et al*., 2012). It is obvious that in the strain AL8 the formation of catechol was predominant, while in strains AMA1102 and AL9 hydroquinone hydroxylase acted simultaneously with phenol hydroxylase. The phenol-hydroxylase test that we employed does not allow to distinguish between *ortho*- and *para*-hydroxylation.

The differences observed in the degradation rates of the investigated strains corresponded to the catechol 1,2-dioxygenase activities. The decisive role of this ring-cleaving enzyme in the strains' capacity for phenol degradation has been observed in other studies as well (Vaillancourt *et al*., 2006; Nair *et al*., 2008). In agreement with this conclusion are the high levels of this enzyme's activities found in the cells of *A. fumigatus* strain AL8, the fastest of the three strains in the degradation of 0.5 g/l phenol as a sole carbon and energy source in the culture medium. The catechol 1,2-dioxygenase activities were lower in all three strains after 72 h of cultivation.

Sequence analyses of putative phenol hydroxylase genes

Six pairs of oligonucleotide primers were designed on the basis of the DNA sequence of the phenol hydroxylase gene of *Aspergillus fumigatus* Af293 (NCBI Acc. No. XM_743491.1) (Nierman *et al*., 2005). All PCR products obtained from genomic DNA of the three strains were electrophoretically analysed and found to have identical profiles (Fig. 4). The results indicate that the hypothetical genes for phenol hydroxylase are of identical length in all three strains.

The primers used were designed such that the resulting fragments overlap. After sequencing of all obtained fragments, two PCR products (lane 1, 705 bp; lane 6, 726 bp) were selected and combined to create a joint DNA sequence of the phenol hydroxylase gene of each of the strains. The sequence of *Aspergillus fumigatus* AL8 (NCBI Acc. No. JQ639073.1–1246 bp DNA) and the sequence of *Aspergillus fumigatus* AL9 (NCBI Acc. No. JQ639074.1–1216 bp) were compared by BLAST analysis with the sequences in the NCBI database. There was 99% identity with the corresponding *Aspergillus fumigatus* Af293 sequence (NCBI Acc. No. XM_743491.1). A single intron consisting of 88 bases was identified in both derived sequences. Sequencing of the phenol hydroxylase gene of *Aspergillus fumigatus* AMA1102 is in progress and the sequence has not yet been registered in the NCBI database.

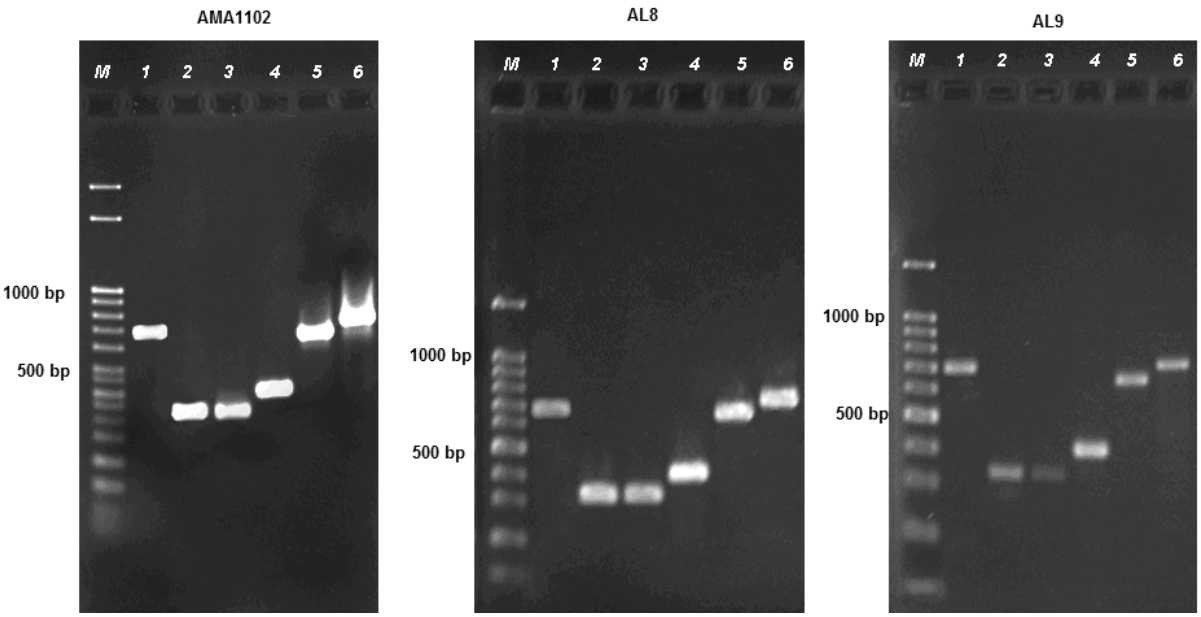

Fig. 4. DNA fragments obtained by PCR with genomic DNA from *A. fumigatus* AMA1102, AL8, and AL9 using the primers given in Table I: lane M, DNA ladder 3 kb and 1.5 kb; lane 1, pAF1f and pAF2r; lane 2, pPHF3 and pPHF4; lane 3, pPHF5 and pAF4; lane 4, pPHF5 and pPHF6; lane 5, pPHF3 and pAF4; lane 6, pPHF3 and pPHF6.

Comparative analysis of the sequences in the NCBI database did not reveal a significant similarity with other earlier reported similar nucleotide sequences obtained from *Aspergillus* strains which could suggest that the phenol hydroxylase-coding genes have undergone significant evolutionary divergence in that genus.

The corresponding protein sequences were 97.4% identical which was due to differences in the fragment size – the sequence obtained for strain AL8 consisted of 385 amino acids while the sequence obtained for strain AL9 consisted of 375 amino acids. The difference resulted from an N-terminal extension which could not be included in the comparison. In fact, the remaining sequences appeared to be identical, so the sequence of AL9 may simply be incomplete (Fig. 5). The protein sequence (NCBI Acc. No. AFJ97046.1) was used in the further comparative analyses.

The protein sequence comparison by the BLASTP 2.2.26+ program revealed 99% identity between both the reference protein sequence, *i.e.* the phenol hydroxylase of *A. fumigatus* Af293, and the respective protein sequence of *A. fumigatus* AL8. The similarities established with other members of flavin adenine nucleotide (FAD)-dependent monooxygenases (FMO) varied between 36 and 45%. This finding confirms the observation that despite the same overall folding typical for flavin-containing aromatic hydroxylases, they can have significant differences in their amino acid sequences (Kalin *et al*., 1992; Enroth *et al*., 1998).

The sequences of two motifs, *i.e*. GXGXXG [for the FAD/NAD(P)H-dependent oxidoreductase] and a highly conserved GD sequence, that interacts with the ribose moiety of FAD, indicated the existence of FAD-binding regions of FMOs (Nakamura *et al*., 2012). The second FAD-binding region was detected in the obtained protein sequences. Despite of the considerable differences in the amino acid sequences, the alignment of putative FAD-binding regions of other reported fungal enzymes with phenol hydroxylase activity revealed a high degree of conservation of this region (Fig. 6). The motif typical for the first binding region was not found due to the incomplete N-terminal end of the gene sequence presented in this work. This motif is generally found in a region flanking the N-terminal end (Nakamura *et al*., 2012).

There is a general agreement that Antarctica is one of the best preserved and clean areas of the planet. An interesting question is how the fungal strains reached Antarctica and what their role is in maintaining the clean environment of the area.

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AL8     1 ERGGIDAAIHSGRDGERDTEPVLTGEEGSLKTIRAKYVIGSDGAHSWVRR  50
                  |||||||||||||||||||||||||||||||||||||||||||
AL9     1 ----------SGRDGERDTEPVLTGEEGSLKTIRAKYVIGSDGAHSWVRR  40

AL8    51 WLGFEMEGDSTNAVWGVVDAILDSDFPDFRRHCTILSQHGTILSVPRENG 100
          ||||||||||||||||||||||||||||||||||||||||||||||||||
AL9    41 WLGFEMEGDSTNAVWGVVDAILDSDFPDFRRHCTILSQHGTILSVPRENG  90

AL8   101 MTRLYVQLPDSMKDICLTDAAQVVKIMAVARRSLFPYTLEYSYCDWWTIY 150
          ||||||||||||||||||||||||||||||||||||||||||||||||||
AL9    91 MTRLYVQLPDSMKDICLTDAAQVVKIMAVARRSLFPYTLEYSYCDWWTIY 140

AL8   151 RVGRRVANHFTYKQRVFLGGDAVHTHTPKGGQGMNVSMQDAYNLGWKLGG 200
          ||||||||||||||||||||||||||||||||||||||||||||||||||
AL9   141 RVGRRVANHFTYKQRVFLGGDAVHTHTPKGGQGMNVSMQDAYNLGWKLGG 190

AL8   201 VLRGQLRPSVLATYESERRPVAQDLIKLDTSMGRVLAGETMSETPEVLQV 250
          ||||||||||||||||||||||||||||||||||||||||||||||||||
AL9   191 VLRGQLRPSVLATYESERRPVAQDLIKLDTSMGRVLAGETMSETPEVLQV 240

AL8   251 YEQLRNYGSGANICYSPNILVASPQQSQQHLAAHLRLGMRFPSHPVVNLA 300
          ||||||||||||||||||||||||||||||||||||||||||||||||||
AL9   241 YEQLRNYGSGANICYSPNILVASPQQSQQHLAAHLRLGMRFPSHPVVNLA 290

AL8   301 SAITMESQSLLPSNGSWRLWVFAGNVVACPAQLKRVNSLGEKLCALTARL 350
          ||||||||||||||||||||||||||||||||||||||||||||||||||
AL9   291 SAITMESQSLLPSNGSWRLWVFAGNVVACPAQLKRVNSLGEKLCALTARL 340

AL8   351 AALQMLSTPFLEILLLYKGRVEEMEVSDFHPIFTR 385
          |||||||||||||||||||||||||||||||||
AL9   341 AALQMLSTPFLEILLLYKGRVEEMEVSDFHPIFTR 375
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Fig. 5. Aligned protein sequences derived from the phenol hydroxylase gene of *Aspergillus fumigatus* AL8 and *Aspergillus fumigatus* AL9, respectively.

Fig. 6. Alignment analysis of the second putative FAD binding regions in fungal phenol hydroxylases. 100% conserved amino acids are coloured black. 1, *A. fumigatus* AL8 (this study); 2, *A. fumigatus* Af293 (XM_743491.1); 3, *A. awamori* NRRL 3112 (GQ279378.1); 4, *Trichosporon cutaneum* ATCC 46490 (L04488.1); 5, *Neosartorya fischeri* NRRL 181 (XP_001265717.1); 6, *Phanerochaete chrisosporum* ATCC 34541 (Nakamura *et al.*, 2012).

There are interesting theories about the spread of various microorganisms and their exceptional survival in harsh conditions (Arenz and Blanchette, 2009; Bridge and Spooner, 2012). Some researchers have examined this issue in historical perspective. It is believed that the old wooden ships reaching this area could cause local fuel contamination as well as some shipwreck could have left mostly wooden debris, and this in turn created a broth for microorganisms with specific features, such as degradation of aromatic compounds (Hughes *et al.*, 2007). On the other hand, the spores of microorganisms, such as molds, which are extremely resistant to unfavourable living conditions, could be transmitted by air and water currents in a variety of directions. In addition, an ornithogenic contribution to soil chemistry must be considered. While our study could not possibly contribute an answer to these questions, it has enriched our knowledge of microbial biodiversity in Antarctica.

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