Heterologous expression and biochemical characterisation of the recombinant β-carbonic anhydrase (MpaCA) from the warm-blooded vertebrate pathogen malassezia pachydermatis

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ABSTRACT

Warm-blooded animals may have Malassezia pachydermatis on healthy skin, but changes in the skin microenvironment or host defences induce this opportunistic commensal to become pathogenic. Malassezia infections in humans and animals are commonly treated with azole antifungals. Fungistatic treatments, together with their long-term use, contribute to the selection and the establishment of drug-resistant fungi. To counteract this rising problem, researchers must find new antifungal drugs and enhance drug resistance management strategies. Cyclic adenosine monophosphate, adenylyl cyclase, and bicarbonate have been found to promote fungal virulence, adhesion, hydrolase synthesis, and host cell death. The CO\textsubscript{2}/HCO\textsubscript{3}-/pH-sensing in fungi is triggered by HCO\textsubscript{3}- produced by metalloenzymes carbonic anhydrases (CAs, EC 4.2.1.1). It has been demonstrated that the growth of M. globosa can be inhibited in vivo by primary sulphonamides, which are the typical CA inhibitors. Here, we report the cloning, purification, and characterisation of the β-CA (MpaCA) from the pathogenic fungus M. pachydermatis, which is homologous to the enzyme encoded in the genome of M. globosa and M. restricta, that are responsible for dandruff and seborrhoeic dermatitis. Fungal CAs could be thus considered a new pharmacological target for combating fungal infections and drug resistance developed by most fungi to the already used drugs.

1. Introduction

Malassezia pachydermatis, originally named Pityrosporum pachydermatis since isolated from the scales of an Indian rhinoceros (Rhinoceros unicornis) with exfoliative dermatitis, is one of the cutaneous commensals in all warm-blooded animals\textsuperscript{1,2}. This opportunistic commensal has the potential to become a pathogen if the skin microenvironment or host defences are altered\textsuperscript{1}. M. pachydermatis relevance has been recognised in both veterinary and human medicine\textsuperscript{3}. Generally, M. pachydermatis is related to otics externa and seborrhoeic dermatitis in dogs, cats, and wild animals\textsuperscript{3}. Although the Malassezia species, such as M. furfur, M. sympodialis, M. globosa, M. obtusa, M. restricta and M. slooffiae are lipid dependent, M. pachydermatis\textsuperscript{4} is the only species that does not require lipids for its growth\textsuperscript{1,2}. Human skin is commonly colonised by lipid-dependent Malassezia yeasts but rarely by M. pachydermatis\textsuperscript{5}. However, M. pachydermatis, together with M. furfur and M. sympodialis, have been isolated from bloodstream infections, contributing to fungemia in hospitalised and severely immunocompromised patients, such as preterm neonates, cancer patients, or patients with pulmonary distress\textsuperscript{6}.

Azoles and the polyene amphotericin B (Amb) are often used to treat Malassezia-related illnesses in humans and animals\textsuperscript{5,6}. Topical antifungal medications, mainlyazole compounds, can effectively treat localised skin lesions. Still, severe cutaneous problems or fungal infections in the lung spreading throughout the whole body can require the use of triazoles drugs, like itraconazole (ITZ) or fluconazole (FLZ)\textsuperscript{6,7}. Unfortunately, the fungistatic properties of azoles and their derivatives, coupled with the continuous usage in the treatment of fungal infections, aided in selecting and establishing drug-resistant fungus strains\textsuperscript{5,6}. The discovery of novel antifungal medications and the improvement of therapeutic strategies to combat drug resistance are required to address and overcome this challenge.

Intriguing, pathogenic and opportunistic fungi can sense changes in the environmental CO\textsubscript{2} levels, which influence the fungal virulence or their environmental survival fitness\textsuperscript{8,9,10}. As a result, the fungal CO\textsubscript{2}-sensing represents a promising target for drugs since controlling fungal differentiation and expression of proteins required for virulent or non-virulent qualities may be pharmacologically relevant. As demonstrated in many fungi, the fungal CO\textsubscript{2}-sensing is governed by bicarbonate (HCO\textsubscript{3}-), which promotes meiosis and sporulation\textsuperscript{9}; and by adenylyl cyclase (AC) as well as cyclic adenosine monophosphate (cAMP), which are involved in spore production\textsuperscript{5,10,11}. In Candida albicans, AC, cAMP, and HCO\textsubscript{3}- have been shown to stimulate filamentous structures (hyphae) needed for fungal virulence, adhesion, production of hydrolases, and inducing cell death in the hosts\textsuperscript{5,6,12,13}. Thus, AC, cAMP
signalling, and CO₂/HCO₃⁻ sensing were suggested as essential elements modulating fungal metabolism and pathogenicity⁶.

The fungal CO₂-sensing, related to the CO₂/HCO₃⁻/pH-sensing, is directly triggered by HCO₃⁻ generated from the action of metalloenzymes known as carbonic anhydrases (CAs, EC 4.2.1.1). CAs represent a superfamily of ubiquitous enzymes catalysing a fundamental reaction for all living organisms, the reversible hydration of CO₂ to HCO₃⁻ and H⁺ (CO₂ + H₂O ⇌ HCO₃⁻ + H⁺)¹⁴-²⁰. Up to date, eight CA gene families or classes have been identified and named with the letters of the Greek alphabet (α, β, γ, δ, ε, η, θ, ψ)¹⁴-¹⁸. In the fungal kingdom, the typical class is represented by β-CAs, and most fungi encode at least one member of this subfamily of enzymes¹¹,²¹,²². In contrast, in some filamentous ascomycetes it is possible to find genes also encoding for β-CAs¹¹,²¹,²².

It has been demonstrated that primary sulphonamides, typical CA inhibitors (CAIs), can inhibit the growth of M. globosa in vivo when the CO₂ availability is limited (i.e., the skin surface infected by the fungus)²³. The genome of the fungal parasite M. globosa, the etiologic agent of specific skin diseases such as pityriasis versicolor, seborrhoeic dermatitis scalp and dandruff, contains a single gene encoding a β-CA (acronym MgpCA). The enzyme showed an appreciable CO₂ hydrase activity, with a $k_{cat}$ value of $9.2 \times 10^{-3}$ s⁻¹ and $k_{cat}/K_{M}$ value of $8.3 \times 10^{-7}$ M⁻¹ s⁻¹²⁴-3⁰. Primary sulphonamides resulted in excellent in vitro inhibitors ($K_I$–63–174 nM)²⁴-­⁰. In contrast, other CA inhibitors such as inorganic anions, diiodocarbamates, monothiocarbamates, phosphonamides, and phenols showed the $K_I$ values in the μM range²⁶-­²⁸,³¹. Subsequently, our groups investigated the biochemical properties and the sulphonamide inhibition profiles of the CA (MreCA) encoded by the genome of M. restricta²²,³₃. This fungus is involved in starting the disequilibrium between the commensals Cutibacterium acne (formerly named Propionibacterium acne) and Staphylococcus sp., both of which contribute to dandruff and seborrhoeic dermatitis symptoms³⁴. MreCA showed a high catalytic activity for the hydration of CO₂ into bicarbonate and protons ($k_{cat}$ value = $1.06 \times 10^{9}$ s⁻¹ and $k_{cat}/K_{M}$ value = $1.07 \times 10^{8}$ M⁻¹ s⁻¹)³². Besides, primary sulphonamide inhibitors inhibited the enzyme with a $K_I$ values <1.0 μM³³.

In this article, we continue our research on fungal CAs, reporting the cloning, purification, and characterisation of the β-CA (MpaCA) from the pathogenic fungus M. pachydermatis, whose CA is homologous to MgCA and MreCA. We should stress that fungal CAs are proposed as potential biomolecules involved in the life cycle of the fungi. Thus, they could represent a new drug target for fighting the fungal infection as well as the drug resistance developed by Malassezia species or other fungi versus the drug compounds clinically used today.

### 2. Materials and methods

#### 2.1. Bacterial strains, vectors, and chemicals

*Escherichia coli* DH5α cells (Agilent, USA) were used for initial cloning, while *E. coli* BL21 (DE3)pLysS cells (Agilent, Santa Clara, CA, USA) were utilised for the heterologous expression of the recombinant *M. pachydermatis* β-CA. The pET100/D-TOPO vector was purchased from Invitrogen (Carlsbad, CA) with the feature to express the recombinant protein as a fusion protein with a 6-histidine tag at the N-terminus. Luria Bertani Broth (LB), ampicillin, and other chemicals were obtained from Merck (Darmstadt, Germany).

#### 2.2. Protein database screening

*M. pachydermatis* β-CA has been identified using the NCBI-BLASTP program, a sequence analysis tool specifically designed to search protein databases. M. globosa β-CA was used as a query sequence to screen protein databases. The output file generated by the NCBI-BLASTP identified an amino acid sequence with the following accession number XP_017991749 (NCBI Reference Sequence), showing a high level of identity with respect to the homologous enzyme. The *Malassezia pachydermatis* β-CA was indicated with the acronym MpaCA.

#### 2.3. Sequence analysis

The program MUSCLE, which was created for performing the multiple alignment of protein sequences, has been used to align the primary structure of all proteins here considered.⁰⁸

#### 2.4. Phylogenetic analysis

The program NGPhylogeny has been run to obtain a phylogenetic dendrogram, searching for the tree with the highest probability.⁰⁸

#### 2.5. Synthetic gene and cloning

The synthetic MpaCA gene was designed in our labs and produced by Life Technologies (Invitrogen, Carlsbad, CA), which is specialised in gene synthesis. The MpaCA gene contained Ndel and Xhol restrictions sites at the 5′- and 3′-ends, respectively, and four base-pair sequences (CACC) necessary for directional cloning at the corresponding 5′-end of the MpaCA gene. The synthetic MpaCA was ligated into the expression vector pET100/D-TOPO (Invitrogen, Carlsbad, CA) to form the expression vector pET100-D-TOPO/MpaCA, containing a nucleotide sequence encoding for a polypeptide with additional six histidines before the insertion point for facilitating the purification of the target protein. The MpaCA gene integrity and lack of errors in the ligation sites were confirmed by bidirectional automated sequencing.

#### 2.6. Heterologous expression

Competent *E. coli* BL21 (DE3)pLysS (Agilent, Santa Clara, CA, USA) cells were transformed with pET100-D-TOPO/MpaCA. A single colony of transformed *E. coli* BL21 (DE3)pLysS was incubated overnight on a shaking incubator in 10 ml Luria-Bertani broth (LB) medium containing ampicillin (100 μg/ml), at 37 °C with constant agitation (200 rpm). The next day, 5 ml of cultured materials was removed and inoculated in 1 L of LB broth. The culture was grown at an OD₆₀₀nm value of 0.6 under vigorous shaking (200 rpm), at 37 °C. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and 0.5 mM ZnSO₄ was added after 30 min incubation for uptake in the expressed protein. The incubation period continued for additional 3 h, at 37 °C, with shaking at 200 rpm. Then, the bacterial suspension was tested and analysed on 12% SDS-PAGE to verify the overexpression of MpaCA. Sodium dodecyl sulphate SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli using 12% gel.⁰⁹
2.7. Enzyme purification

At 3-h post-induction, cells were harvested and disrupted by sonic- 
ation at 4 °C. Following centrifugation, the supernatant was 
loaded onto HIS-Select HF Nickel Affinity Gel (Sigma-Aldrich, St. 
Louis, MO), equilibrated with 0.02 M phosphate buffer (pH 8.0) 
containing 0.01 M imidazole and 0.5 M KCl, at a flow rate of 
1.0 ml/min. The recombinant MpaCA protein was eluted from the 
column with 0.02 M phosphate buffer (pH 8.0) containing 0.5 M 
KCl and 0.3 M imidazole at a flow rate of 1.0 ml/min. Active frac-
tions (0.5 ml) were collected and combined to a total volume of 
2.5 ml. Subsequently, they were dialysed, concentrated, and ana-
lysed by SDS-PAGE. The protein concentration of the purified 
recombinant enzyme was determined with a Bio-Rad protein assay 
based on the Bradford method40. At this stage of purification, the 
enzyme was at least 95% pure, and 1.0 mg of the total recombin-
ant enzyme was obtained from 1 L of bacterial culture.

2.8. Enzyme protonography

For protonography, SDS-PAGE was performed as described by De 
Luca et al.41. Samples were mixed in a loading buffer without 2-
mercaptoethanol, and they were not boiled to avoid protein 
denaturation. After electrophoresis, the gel was subject to proto-
noigraphy to detect the hydratase activity41.

2.9. Enzyme assay

An applied photophysics stopped-flow instrument has been used for 
assaying the CA catalysed CO₂ hydratation activity42. Phenol red 
(at a concentration of 0.2 mM) has been used as an indicator in a 
buffer containing 20 mM Tris (pH 8.3), 20 mM NaClO₄ (for main-
taining a constant ionic strength), measuring the absorbance max-
umum of 557 nm, and following the initial rate of the CA-catalysed 
tation. For acetazolamide, at least six traces of the initial 5–
10% of the 

2.10. Inhibition assay with acetazolamide (AAZ)

For acetazolamide, at least six traces of the initial 5–10% of the 
reaction have been used for determining the initial velocity. The 
uncatalysed rates were determined in the same manner and 
subtracted from the total observed rates. Stock solutions of inhibi-
tor (10–50 mM) were prepared in distilled-deionized water, and 
dilutions up to 0.01 mM were done thereafter in the assay buffer. 
Inhibitor and enzyme solution were preincubated together for 
15 min, at room temperature, to allow the formation of the E-I 
complex or for the eventual active site mediated hydrolysis of the 
inhibitor. As reported earlier, the inhibition constant values were 
obtained by non-linear least-squares methods using PRISM 3 and 
represents the mean from at least three different determinations43.

3. Results and discussion

M. pachydermatis genome has a 726-bp gene region, encoding a 
CA polypeptide chain of 242 amino acid residues (Figure 1). 
Figure 1 was generated by the NCBI BlastP suite and showed that 
MpaCA contained all the consensus domains, which typify the 
β-CA class (Figure 1). Four amino acid residues of the zinc-binding 
site (C47, D49, H103, C106), thirteen residues of the active site 
cleft (Q38, P40, C47, D49, S50, R51, G63, F66, F88, L93, H103, 
C106, H213) and, as β-CAs are active only as dimers (or other mul-
tiple oligomers, such as tetromers or octamers),44,45 nineteen resi-
dues of the dimer interface (S48, D49, S50, R51, C58, G63, F66, 
L65, V67, R69, V83 S84, T87, F88, H213, I215, H216, G218, L220) 
have been mapped by the BlastP suite on MpaCA (Figure 1).

The encoded fungal enzyme was named MpaCA; it is homologous 
to the β-CAs previously identified by our group in the genome 
of M. restricta and M. globosa, which were annotated as 
MreCA and MgCA, respectively. To show the relevant degree of identity between these enzymes, we aligned MpaCA, MgCA and 
MreCA (Figure 2).

Figure 2 shows that the three enzymes have 173 fully con-
served amino acids. In particular, MpaCA, MreCA, and MgCA have 
three residues (two cysteines and one histidine) totally conserved, 
which are involved in the catalytic mechanism of the enzyme, act-
ing as zinc ligands, and the catalytic dyad (one aspartate and one 
arginine) near the first catalytic cysteine in the polypeptide chain 
(see Figure 2). Above-reported aspartate and arginine residues are 
involvid in activating the zinc-coordinated water molecule 
responsible for nucleophilic attack to the substrate46.

A most parsimonious phylogenetic tree was constructed to 
study the evolutionary links between the Malassezia β-CA and 
similar enzymes identified in the genome of other fungi or

Figure 1. MpaCA amino acid sequence (A) and schematic representation (B) of the β-CA class consensus domains. Legend: lower letter case, MpaCA amino acid sequence; solid black line, polypeptide chain; solid light blue line, β-CA superfamily conserved domain; red triangles, conserved amino acids present at the enzyme’s zinc-binding site; green triangles, conserved amino acids present at the enzyme’s dimer interface; blue triangles, conserved amino acids present at the enzyme’s active site.
different organisms belonging to other taxa, such as insects, plants, fungi, algae, and bacteria. As a result, a radial dendrogram has been generated, which is reported in Figure 3. The Malassezia enzymes are closely related to each other and are phylogenetically very close to β-CAs from the pathogenic fungi *Ustilago maydis* and *Cryptococcus neoformans*. Intriguingly, the Malassezia β-CA cluster is well-separated from the other β-CAs identified in species of fungi different from Malassezia (Figure 3). We postulated that this is the result of a gene duplication event that occurred many millions of years ago throughout the history of these organisms.
of the fungal β-CA gene, which separated the Malassezia β-CAs from those of other fungi, except Cryptococcus neoformans and Ustilago maydis (Figure 3).

Our research on fungal β-CAs from Malassezia species prompted us to produce the recombinant MpaCA to compare its biochemical properties with those obtained for other two homologous Malassezia enzymes, MgCA and MreCA. The electropherogram developed by the SDS-PAGE of the fungal β-CA shows that MpaCA has been purified to homogeneity (Figure 4). The MpaCA monomer had an apparent molecular mass of about 30 kDa. The recombinant enzyme fused to the His-tag tail was expected to be 31 kDa.

The SDS–PAGE was also used to examine the MpaCA hydratase activity. In this case, MpaCA was loaded onto the polyacrylamide gel and subjected to protonography for detecting the ions (H+) caused by the MpaCA-catalysed conversion of CO2 into bicarbonate and protons. The pH variation due to the CO2 hydration reaction has been visualised as a yellow band in the protonogram, i.e., the electropherogram developed following the corresponding experimental protocol46. According to the results displayed in Figure 4, MpaCA resulted as an active enzyme migrating with a molecular mass of 30 kDa.

These results prompted us to investigate the MpaCA kinetic parameters using CO2 as substrate and the stopped-flow spectrometry as the technique allowing the study of fast reactions in solution. Table 1 shows a comparison of the kinetic parameters of recombinant MpaCA with those of MgCA and MreCA counterparts; the latter proteins were cloned as 6-histidine tag fusion polypeptides like MpaCA. For comparative purposes, we have also included two β-CAs from Homo sapiens, namely the isoform I (hCA I) and II (hCA II).

MpaCA showed a significant catalytic activity, with a $k_{\text{cat}}$ value of $3.8 \times 10^3$ s$^{-1}$ and a $k_{\text{cat}}/K_M$ value of $9.7 \times 10^5$ M$^{-1}$ s$^{-1}$. The data reported in Table 1 demonstrate that MreCA has a catalytic activity ($k_{\text{cat}}$ value of $1.06 \times 10^6$ s$^{-1}$ and $k_{\text{cat}}/K_M$ value of $1.07 \times 10^8$ M$^{-1}$ s$^{-1}$) higher than that of MpaCA and MgCA, being in the same order of the human isoform hCA II, which is considered among the fastest CA known so far. Although MreCA, MpaCA, and MgCA have 173 fully conserved amino acids within a 242 amino acids-long polypeptide chain, they exhibited a pronounced difference in their inhibition behaviour with respect to the classical primary sulphonamide inhibitor acetazolamide (AAZ). MpaCA displayed an inhibition constant ($K_i$) value of 623 nM, which resulted only 2.5 times higher than that of hCA I ($K_i$ value = 250 nM) (Table 1). Instead, the MreCA activity was highly inhibited by AAZ, with an inhibition constant of 50.7 nM, while MgCA was slightly sensitive to AAZ inhibition ($K_i$ value = 74,000 nM).

These findings could pave the way for developing highly selective drugs that do not interfere with human skin integrity by inhibiting the β-CAs encoded by healthy scalp microbes, which probably are well inhibited by AAZ as the other bacterial β-CAs, and avoiding interference with human CAs since mammals only contain α-CAs.

### 4. Conclusions

The ability to enter a host, evade host defences, grow in a host environment, counteract host immune responses, assimilate iron and nutrients from the environment, and perceive environmental change are all prerequisites for microorganism pathogenicity. Many enzymes aid the pathogenicity of the microbes. Among them, it is possible to mention proteases, neuraminidases, phospholipases, and ureases47. Recently, a new superfamily of metalloenzymes, namely CAs, has been identified as biomolecules playing a pivotal role in microbial virulence and pathogenicity48,49. The CO2-sensing, triggered by HCO3$^-$ produced in a CA-dependent manner, is an essential modulator of fungal metabolism and pathogenicity. In this context, we have reported here the cloning, purification, and initial characterisation of MpaCA encoded from the genome of the pathogenic fungus M. pachydermatis, which is the aetiological agent of otitis externa and seborrhoeic dermatitis in dogs, cats, and other wild animals50. The recombinant MpaCA, prepared as 6xHis-tag fusion protein, was efficiently expressed and purified by affinity chromatography. MpaCA showed a significant catalytic activity for the hydration of CO2 into bicarbonate and protons, with the following kinetic parameters: $k_{\text{cat}}$ value of $3.8 \times 10^3$ s$^{-1}$ and $k_{\text{cat}}/K_M$ value of $9.7 \times 10^5$ M$^{-1}$ s$^{-1}$. The enzyme is also sensitive to inhibition by the classical sulphonamide inhibitor acetazolamide ($K_i$ value of 50.7 nM). Intriguingly, although Malassezia β-CAs resulted

### Table 1. Kinetic parameters of MpaCA compared with those of MgCA, MreCA and human isoenzyme hCA I and hCA II (α-class).

| Organism                  | Enzyme Acronym | Class | $k_{\text{cat}}$ (s$^{-1}$) | $K_M$ (mM) | $k_{\text{cat}}/K_M$ (M$^{-1}$s$^{-1}$) | $K_i$ (AAZ) (nM) |
|---------------------------|----------------|-------|-----------------------------|------------|--------------------------------------|-----------------|
| Homo sapiens              | hCA I          | α     | $2.0 \times 10^5$           | 4.0        | $5.0 \times 10^7$                    | 250             |
|                           | hCA II         | α     | $1.4 \times 10^6$           | 9.3        | $1.5 \times 10^8$                    | 12              |
| Malassezia pachydermatis  | MpaCA          | β     | $3.8 \times 10^5$           | 39.7       | $9.7 \times 10^6$                    | 623             |
| Malassezia restricta      | MreCA          | β     | $1.06 \times 10^6$          | 10.1       | $1.07 \times 10^6$                   | 51              |
| Malassezia globosa        | MgCA           | β     | $9.2 \times 10^5$           | 11.1       | $8.3 \times 10^5$                    | 74,000          |

The β-class enzymes were tested for the CO2 hydration reaction in 20 mM Tris buffer pH 8.3 and 20 mM NaClO4 at 25 °C. Reported mean values are from 3 different assays performed by the stopped-flow technique; errors were in the range of ±5–10% of the reported values (data not shown).
phylogenetically close to each other, they showed substantial differences in their inhibition with AAZ as well as in their catalytic constants; in particular, the $k_{cat}$ value of MreCA was an order higher than those of MpaCA and MgCA. Further X-ray crystallographic studies on MpaCA, MreCA, and MgCA with classical CA inhibitors will be helpful in better understanding the inhibitory behaviour of these and other fungal $\beta$-CAs, whose in vivo inhibition might be essential for fighting fungal diseases and the phenomenon of the drug resistance.

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