Cryptococcus neoformans Senses CO₂ through the Carbonic Anhydrase Can2 and the Adenylyl Cyclase Cac1

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Cryptococcus neoformans, a fungal pathogen of humans, causes fatal meningitis in immunocompromised patients. Its virulence is mainly determined by the elaboration of a polysaccharide capsule surrounding its cell wall. During its life, C. neoformans is confronted with and responds to dramatic variations in CO₂ concentrations; one important morphological change triggered by the shift from its natural habitat (0.033% CO₂) to infected hosts (5% CO₂) is the induction of capsule biosynthesis. In cells, CO₂ is hydrated to bicarbonate in a spontaneous reaction that is accelerated by carbonic anhydrases. Here we show that C. neoformans contains two β-class carbonic anhydrases, Can1 and Can2. We further demonstrate that CAN2, but not CAN1, is abundantly expressed and essential for the growth of C. neoformans in its natural environment, where CO₂ concentrations are limiting. Structural studies reveal that Can2 forms a homodimer in solution. Our data reveal Can2 to be the main carbonic anhydrase and suggest a physiological role for bicarbonate during C. neoformans growth. Bicarbonate directly activates the C. neoformans Cac1 adenylyl cyclase required for capsule synthesis. We show that this specific activation is optimal at physiological pH.

Cryptococcus neoformans is an opportunistic fungal pathogen which causes potentially life-threatening meningoencephalitis in humans worldwide (27). This ubiquitous basidiomycete is acquired by inhalation and infects mainly immunocompromised individuals, particularly those with AIDS (1). Upon inhalation, spores reach the alveolar space and disseminate via the blood to infect the central nervous system (6). In order to cause infection, C. neoformans synthesizes various virulence factors, including those allowing the ability to grow at 37°C, melanin, phospholipase, urease, and most importantly, the prominent polysaccharide capsule surrounding the cell wall (13). This structure not only provides a protective, physical barrier, but it can also interfere with phagocytosis and thus clearance of the fungus by the immune system (6). Among the four capsular serotypes (A, B, C, and D), serotype A accounts for >90% of clinical isolates and >99% of isolates from AIDS patients (4).

As an opportunistic pathogen, C. neoformans is exposed to very different environmental conditions during its infectious life cycle. One major variable is the composition of its surrounding atmosphere. During growth in its natural habitat, such as eucalyptus trees or soil contaminated by pigeon droppings, it is exposed to relatively low ambient CO₂ concentrations (0.033%). In contrast, upon inhalation and subsequent infection, it must adapt to the elevated levels (5%) of CO₂ found in its mammalian host. These dramatically different conditions have a profound influence on C. neoformans morphology. For example, a physiological concentration of CO₂ (i.e., 5%) promotes capsule biosynthesis (10).

The involvement of CO₂ in virulence has also been reported for other microbial pathogens. For example, transcription of the virulence genes emm and scp4 is activated in response to CO₂ in group A streptococci, and elevated concentrations of CO₂ stimulate germination, capsule biosynthesis, and toxin production in Bacillus anthracis (18, 20). CO₂ at a physiological level is also an important differentiation signal in the pathogenic fungus Candida albicans (14, 32). In this ascomycete, CO₂ induces filamentation, a morphological change that represents the pathogen’s most prominent virulence determinant.

CO₂ is spontaneously hydrated to bicarbonate (HCO₃⁻), but this chemical reaction is accelerated by carbonic anhydrases (CAs). CAs are zinc metalloenzymes classified as three main evolutionarily independent classes, as follows: all mammalian CAs belong to the α class; β-class CAs are found in plants, algae, bacteria, and archaea; and the γ class so far consists of a single enzyme identified in the methanoarchaeon Methanosaurococcus thermophilus (33, 34). We have previously characterized the C. albicans β-class CA and established its function as a CO₂ scavenger essential for pathogenesis in niches where the available CO₂ is limited (14). Likewise, Saccharomyces cerevisiae has recently been shown to synthesize a CA whose activity increases at low CO₂ concentrations (3).
TABLE 1. Primers used for cloning of CAN1 and CAN2

| Name          | Sequence (5'-3')                                                                 |
|---------------|---------------------------------------------------------------------------------|
| CnCAN1f                                  | ATGACCCATTCTAGTCTCGGAAT                                                          |
| CnCAN1b                                  | CATAGAAATGTTATATTTT                                                             |
| CnCAN1br                                 | AAGACATTGACCTTCACAAAC                                                           |
| CnCan1bf                                | GGAATTTTCTCCTCTTGAC                                                            |
| GCAN1r                                  | ATGTATTATCTTGCAGTCG                                                            |
| GCAN1f                                  | TGATGATTAGTTTTTG                                                                |
| GCAN1f2                                 | TATTTTGCAATCCTGGAATG                                                            |
| CAN11fG                                 | ATGTTATATTCCTTCATGGA                                                             |
| GCAN1f                                  | TTTTTGCATACCTGGGACAT                                                             |
| GCAN1f2                                 | TTTTTGCATACCTGGGACAT                                                             |
| CnCAN2r                                 | ATGAGCCATTCTAGTCTCGGAAT                                                          |
| CnCan2f                                 | CATAGAAATGTTATATTTT                                                             |
| GCAN1f                                  | TTTTTGCATACCTGGGACAT                                                             |
| CnCAN2r2r                               | GGAATTTGCAATCCTTCAGTG                                                           |
| CnCAN2r2f                               | GGAATTTGCATTCTTGCAGTCG                                                          |
| CnCACAN2r2f                             | GAATTTCATCGTCGAGGACCTCGGTC                                                     |

In C. neoformans, the Gap1-cyclic AMP (Gap1-cAMP) signaling pathway regulates capsule biosynthesis (2). Although no known links have been established between heterotrimeric G proteins and CO2 chemosensing, we have previously demonstrated that physiological levels of bicarbonate directly stimulate the C. neoformans adenyl cyclase (AC) Cac1 (14). This finding revealed that fungi, which possess only a single AC, appear to retain the functionality of both families of mammalian ACs, which are the G-protein-regulated-like transmembrane ACs and the bicarbonate-responsive-like soluble ACs (14).

For this study, we investigated the role of carbonic anhydrase and adenyl cyclase in the CO2-sensing pathway of C. neoformans. We demonstrate that the Ca Can2 is essential for the survival of C. neoformans serotype A in its natural environment. We give evidence that bicarbonate regulation of the adenyl cyclase Cac1 allows the pathogen to detect and respond to changes in CO2 concentrations in its environment and that the bicarbonate stimulation of Cac1 is maximal at physiological pH.

MATERIALS AND METHODS

Strains, media, and growth conditions. C. neoformans KN99 (MATa, serotype A) (25), used throughout this study, was cultured in yeast extract-peptone-dextrose (YPD) medium at 37°C. Escherichia coli strain BL21 (Invitrogen, Paisley, United Kingdom) was used for protein production. Bacterial strains were routinely maintained at 37°C in LB medium. When required, media were solidified with 2% agar and supplemented with carbenicillin (100 µg/ml). Complementation experiments with CAN2 and CAC1 were performed using E. coli strain EDCM636 (19) and the C. albicans cdc35 by PCR using specific primers. The PCR conditions to amplify the 10 min, and the reverse transcription step at 37°C was extended to 2 h. The triphosphates. The first incubation step of the mixture was performed at 70°C for 10 min.

Cloning and expression of CAN1 and CAN2. All primers used to clone CAN1 and CAN2 are indicated in Table 1. Total RNA was extracted using a QIAGEN RNeasy kit (QIAGEN, Crawley, United Kingdom) and reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen) following the manufacturer’s recommendations, except that 5 µg total RNA was incubated with 50 pmol of gene-specific reverse primer and 2 nmol of deoxynucleoside triphosphates. The first incubation step of the mixture was performed at 70°C for 10 min, and the reverse transcription step at 37°C was extended to 2 h. The cDNA was purified using a QIAquick purification kit (QIAGEN) and amplified by PCR using specific primers. The PCR conditions to amplify CAN2 were 94°C for 2 min, 30 cycles at 94°C for 30 s and 58°C for 1 min 30 s, and a final step at 72°C for 1 min 30 s. Cloning of CAN1 was attempted by reverse transcription using the primers CnCAN1f, CnCAN1b, GCAN1f, GCAN1r, CAN1f1G, CAN1f2G, CnCAN1r, CnCAN1br, and GCAN1r in different combinations (Table 1). For expression of the Can2 protein, the coding region of CAN2 was amplified using the primers CnCACAN22f and CnCACAN22r (Table 1). An in-frame fusion of glutathione S-transferase (GST)—Can2 was obtained by ligating the coding region of CAN2 as a BamHI-EcoRI fragment to pGEX-6P-2 (Amersham Biosciences, Little Chalfont, United Kingdom). The resulting construct was introduced into E. coli BL21 cells.

Can2 purification and enzymatic analysis. Can2 was purified as a GST fusion protein using sepharose-4B columns (Amersham Biosciences) according to the manufacturer’s instructions. Protein activity was assessed by the electrophoretic method of Wilbur and Anderson (36), as further developed by Sigma (Sigma-Aldrich, Gillingham, United Kingdom). For this assay, the enzyme (50 µl) was diluted in a buffer at pH 8.3 and mixed with CO2-saturated water (3:2 [vol:vol]). The time needed for the pH of the mixture to drop to 6.3 was measured and compared with that for a blank that contained no enzymes. Enzyme activity was calculated using the following formula: activity = (blank time – assay time)/(assay time × 0.005). Bovine carbonic anhydrase (Sigma-Aldrich) and purified GST were used as positive and negative controls, respectively. Inhibition experiments were performed in the presence of 1 to 1,000 nM ethoxyzolamide (Sigma-Aldrich) dissolved in 5% dimethyl sulfoxide (VWR, Poole, United Kingdom).

For analysis of its glycosylation state, GST-Can2 was cleaved with Precision protease (Amersham Biosciences), and GST was removed by incubation with glutathione-Sepharose 4B. Can2 was concentrated and subjected to size-exclusion chromatography on a Superdex 200 column (Amersham Biosciences) in 20 mM Tris, pH 7.8, and 25 mM NaCl. A gel filtration standard containing proteins with molecular masses of 670 kDa, 158 kDa, 44 kDa, 17 kDa, and 1.35 kDa (Bio-Rad, Hercules, CA) was used as a reference.

Cloning and expression of a truncated form of the C. neoformans adenyl cyclase containing gene CAN1. A truncated form of the open reading frame of the C. neoformans CAC1 gene containing the putative catalytic domain (amino acids 1825 to 2271) was amplified by PCR, using cdNA as a template, and cloned into pEF-B (24) as a PstI-BamHI fragment. The subsequent vector was linearized with NheI and introduced into strain CR276 of C. albicans by using lithium acetate (28).

Calculation and AC activity. The fragment encoding amino acids 1825 to 2271 of C. neoformans Cac1 was cloned into pGEX-6P-2 as a BamHI-EcoRI fragment. GST-Cac1 was expressed in E. coli strain BL21 and purified using glutathione-Sepharose 4B columns (Amersham Biosciences) according to the manufacturer’s instructions. Cyclase assays were performed in a final volume of 100 µl, using ~100 ng of purified GST-Cac1 fusion protein in the presence of 50 mM Tris-HCl (pH 7.5), 10 mM ATP, 10 mM MgCl2, and the indicated concentrations of NaHCO3 (see Fig. 9A). Reaction mixtures were incubated at 30°C for 30 min and stopped by the addition of 100 µl of 0.2 N HCl. The cAMP formed was measured as previously described, using a Correlate-EIA Direct CAMP enzyme immunoassay kit (Assay Designs) (11, 14), and data analyses were performed using GraphPad Prism 4.0a. Cyclase activity in response to pH was measured as described above, except that the assay was performed in the absence of 8 mM NaHCO3 at the indicated pHs (Fig. 9B).

Modeling of the Can2 homodimer. The amino acid sequence of Can2 was duplicated and aligned with the sequences of CAs from E. coli (PDB entry 1T75, chains D and E) and Porphyromonas purpureus (PDB entry 1DDZ, chain B) by using ClustalW (7) and Genedoc (http://www.psc.edu/biomed/genedoc). A homology model for Can2 was generated from these templates by using Modeller (29). The Can2 model and surfaces were visualized and analyzed using the programs Pymol (DeLano Scientific; http://www.pymol.org) and Py-thon Molecular Viewer (30), and amino acid conservations were calculated with Consurf (9).

Growth inhibition of C. neoformans by ethoxyzolamide. C. neoformans was grown in liquid YPD medium at 37°C for 16 h. Serial 10-fold dilutions (10 to 10 cells) were spotted onto YPD agar containing 3 mM ethoxyzolamide dissolved in 4% dimethyl sulfoxide, incubated in 0.03% or 5% CO2 for 20 h, and screened for growth. Controls consisted of C. neoformans cells grown under the same conditions in YPD containing 4% dimethyl sulfoxide.

Disruption of C. neoformans CAN1 and CAN2 genes. Gene disruptions were carried out as previously described (22, 23). The disruption cassette was constructed by PCR fusion, using a strategy similar to that used by Kawaiyama and colleagues (15). Upstream and downstream regions flanking CAN2 and the NAT1 marker (17) were PCR amplified using an HPFCR kit from Clontech (Palo Alto, CA) and the primers CAN2-5′, CAN2-3′, MKRCAN2f, and MKRCAN2r (Table 2). In addition, 5 ng of each of the three gel-purified amplicons was used as a substrate for PCR fusion with the primers CAN2-5′ and CAN2-3′, under the following conditions: 94°C for 30 s and 35 cycles at 94°C for 15 s and 68°C for 4 min. The amplicon obtained, representing the can2Δ::NAT1 allele, was used to transform strain KN99 by biolistic DNA delivery.
ery, and transformants were selected on YPD containing 100 μg/ml neomycin (Werner BioAgents, Jena, Germany). CAN1 was disrupted similarly to CAN2, using the primers CAN1ex, CAN1-5', CAN1-5', MKRicCAN1, CAN1-3', MKRicCAN1, CAN1-3', and CAN1ex2 (Table 2). Integration of the cassette was confirmed by PCR, using a primer that anneals to a region outside the disruption cassette (CAN2ex) (Table 2) and a primer that anneals to a sequence within the marker (MATa1::NAT1) (Table 2). Twenty colonies were screened, and three putative homologous integrants were identified. A second pair of primers was used to confirm the presence of the cassette (CAN2ex2-NAT1R) (Table 2). PCR amplification and restriction of the colonies were screened, and three putative homologous integrants were identified through a database search using the amino acid sequences of the CAs from C. albicans and C. cerevisiae (16). Sequence alignments (Fig. 1) assigned Can1 and Can2 to the β class of CAs, containing one conserved histidine, one aspartate, and two cysteine residues that function as zinc ligands (31, 34). Quantitation of the relative expression of CAN1 and CAN2 by reverse transcription-PCR revealed that only CAN2 was highly expressed under standard laboratory growth conditions (data not shown). Sequence analysis of the cDNA revealed that CAN2 contains a 720-bp coding region. Purified Can2 shows carbonic anhydrase activity that is specifically inhibited by ethoxzolamide. E. coli contains a functional CA encoded by the can gene (19). Deletion of this gene leads to a growth defect that is manifested in air but can be complemented with 5% CO2. The expression of CAN2 in the E. coli CA mutant EDCM636 complemented its growth defect in air (Fig. 2A), showing that CAN2 encodes a functionally active CA and that this protein can be expressed by a prokaryotic organism. Can2 was purified as a GST fusion protein. Sodium dodecyl sulfate-polyacrylamide gel electrophore-

| Name               | Sequence (5'-3')                                                                 |
|--------------------|---------------------------------------------------------------------------------|
| CAN1ex             | GAAAAAGCAGACCTGGGCTGTC                                                         |
| CAN1-5'            | CTTCTGACCTCGTGC                                                              |
| CAN1-5'            | GATAATGCTCTGTGGATTACCTTGGC                                                     |
| MKRicCAN1          | CACCCGAACGAAATGCGATTTTTTTCG                                                 |
| CAN1-3'            | ACATTTCAACCTTCGAGGPG                                                          |
| MKRicCAN1          | CAGCTGAAGGAAATGCGATTTTTTTCG                                                   |
| CAN1-3'            | GACTTTGGAATTCGCTGG                                                           |
| CAN1ex2            | TTTCCAGGGTACGAGGAGG                                                          |
| MKRicCAN2          | CACCCGAACGAAATGCGATTTTTTTCG                                                   |
| CAN2ex             | GACTTTGGAATTCGCTGG                                                           |
| MKRicCAN2          | TTTCCAGGGTACGAGGAGG                                                          |
| CAN2-5'            | CTCTTGACCCTCTGC                                                             |
| MKRicCAN2          | CACCCGAACGAAATGCGATTTTTTTCG                                                   |
| CAN2-5'            | ACATTTCAACCTTCGAGGPG                                                          |
| MKRicCAN2          | CAGCTGAAGGAAATGCGATTTTTTTCG                                                   |
| CAN2-3'            | GACTTTGGAATTCGCTGG                                                           |
| MKRicCAN2          | TTTCCAGGGTACGAGGAGG                                                          |
| CAN2ex2            | TTTCCAGGGTACGAGGAGG                                                          |
| MKRicCAN2          | CACCCGAACGAAATGCGATTTTTTTCG                                                   |
| CAN2-5'            | CTCTTGACCCTCTGC                                                             |
| MKRicCAN2          | CACCCGAACGAAATGCGATTTTTTTCG                                                   |
| CAN2-3'            | GACTTTGGAATTCGCTGG                                                           |
| MKRicCAN2          | TTTCCAGGGTACGAGGAGG                                                          |
| MKRicCAN2          | CACCCGAACGAAATGCGATTTTTTTCG                                                   |
| CAN2-5'            | CTCTTGACCCTCTGC                                                             |
| MKRicCAN2          | CACCCGAACGAAATGCGATTTTTTTCG                                                   |
| CAN2-3'            | GACTTTGGAATTCGCTGG                                                           |
| MKRicCAN2          | TTTCCAGGGTACGAGGAGG                                                          |
| CAN2ex2            | TTTCCAGGGTACGAGGAGG                                                          |

**TABLE 2. Primers used for disruption of CAN1 and CAN2**

**RESULTS AND DISCUSSION**

**CAN2 from C. neoformans encodes a highly expressed β-class carbonic anhydrase.** CAN1 and CAN2 of C. neoformans, encoding two putative carbonic anhydrases, were identified through a database search using the amino acid sequences of the CAs from C. albicans and C. cerevisiae (16). Sequence alignments (Fig. 1) assigned Can1 and Can2 to the β class of CAs, containing one conserved histidine, one aspartate, and two cysteine residues that function as zinc ligands (31, 34). Quantitation of the relative expression of CAN1 and CAN2 by reverse transcription-PCR revealed that only CAN2 was highly expressed under standard laboratory growth conditions (data not shown). Sequence analysis of the cDNA revealed that CAN2 contains a 720-bp coding region. Purified Can2 shows carbonic anhydrase activity that is specifically inhibited by ethoxzolamide. E. coli contains a functional CA encoded by the can gene (19). Deletion of this gene leads to a growth defect that is manifested in air but can be complemented with 5% CO2. The expression of CAN2 in the E. coli CA mutant EDCM636 complemented its growth defect in air (Fig. 2A), showing that CAN2 encodes a functionally active CA and that this protein can be expressed by a prokaryotic organism. Can2 was purified as a GST fusion protein. Sodium dodecyl sulfate-polyacrylamide gel electrophore-

**FIG. 1.** C. neoformans CAN1 and CAN2 encode β-class carbonic anhydrases. The figure shows an alignment of sequences of β-class carbonic anhydrases from Saccharomyces cerevisiae, Candida glabrata, Candida albicans, Schizosaccharomyces pombe, Aspergillus nidulans, C. neoformans, and Ostilago maydis. Identical residues are highlighted in dark gray, and conserved amino acids are shown in light gray. The four conserved residues that are important for zinc binding are labeled with asterisks.
sis analysis revealed a single band at 52 kDa, corresponding to the expected molecular mass of GST (26 kDa) added to that of Can2 (26 kDa) (Fig. 2B). The enzymatic activity of the fusion protein was assessed by the electrometric method of Wilbur and Anderson (36). Can2, but not the GST control, displayed carbonic anhydrase activity (Fig. 3A). Can2 exhibited a lower specific activity than that of bovine CA, which reflects the fact that the latter belongs to a different class of CAs (α class) and suggests that the GST tag may affect its activity. The activities of both Can2 and bovine CA were inhibited by ethoxyzolamide (Fig. 3B). Not surprisingly, ethoxyzolamide, which was developed as a specific inhibitor of α-class CAs and only recently tested against β-class CAs (38), displayed a higher affinity for bovine CA than for Can2.

Structural studies of Can2. The basic building block of β-class CAs is a homodimer, but most of these CAs have been reported to form higher-order oligomers through the association of up to four dimers (33). We have found, however, that Can2 exclusively forms homodimers in solution, as judged by size-exclusion chromatography on a Superdex 200 column (Fig. 4). We modeled this Can2 homodimer (Fig. 5A) based on its homology to the CAs from E. coli (8) and Porphyridium purpureum (21). In this Can2 model, an additional N-terminal helix compared to the Pisum sativum CA shields the surface patch exploited by the latter enzyme for oligomerization. The E. coli and P. purpureum CAs, in contrast, use their front faces for higher-order oligomerization. The charge distribution on the surface of the Can2 model reveals only small hydrophobic areas on the front side of the protein, with a larger hydrophobic groove on top of the dimer (Fig. 5B). Hydrophobic surface regions are often shielded from solvents through protein-protein interactions, and this surface part close to the active-site zinc ion might mediate a regulatory heterologous interaction. The high level of conservation of the residues forming and
lining this groove among the closest Can2 relatives also hints at a functional relevance of this surface area (Fig. 5C). Carbonic anhydrases have previously been shown to participate in the formation of protein complexes (26, 35), and it is tempting to speculate that the Can2 surface acts as a binding site for the bicarbonate effector and potential interaction partner Cac1.

**Ethoxyzolamide interferes with growth of C. neoformans.** Since ethoxyzolamide inhibits carbonic anhydrase activity in vitro, we investigated its effect on the growth of C. neoformans. In the presence of 3 mM ethoxyzolamide, the growth of C. neoformans $\left(10^5, 10^4, \text{ and } 10^3 \text{ cells}\right)$ in 0.033\% CO$_2$ was significantly reduced. In addition, we observed, as expected, that 5\% CO$_2$ complemented the growth defect (Fig. 6). These results give evidence that ethoxyzolamide inhibits the activity of intracellular carbonic anhydrase in C. neoformans and that the pathogen requires carbonic anhydrase activity for normal growth in ambient air concentrations of CO$_2$. Since C. neoformans is exposed to limited ambient CO$_2$ concentrations (0.033\%) during the majority of its life cycle and must adapt to high levels of CO$_2$ (5\%) during infection, we hypothesized that Can2, the major carbonic anhydrase, plays an important role in growth and capsule biosynthesis. We investigated this issue by knocking out CAN1 and CAN2 and screening the mutants for the ability to grow in ambient air concentrations of CO$_2$.

**FIG. 4.** Size-exclusion chromatography profile of Can2 on a Superdex 200 column. The arrows indicate elution positions for marker proteins of the indicated molecular masses. The Can2 elution peak is found at the position expected for a 52-kDa homodimer of Can2 monomers (26 kDa).

**FIG. 5.** Homology model for the structure of Can2. (A) Ribbon representation of the modeled homodimeric structure of Can2. The two monomers are colored blue (monomer A) and red (monomer B), respectively. The N and C termini as well as the active-site zinc ions are labeled. (B) Electrostatic surface of the modeled Can2 structure. Blue indicates positive charges, red indicates negative charges, and gray indicates hydrophobic areas. The tilted view reveals a mainly hydrophobic groove on top of the enzyme. (C) Surface of the Can2 dimer colored according to sequence conservation. Blue indicates high amino acid conservation, green indicates medium conservation, and red indicates high variation.
Can2, but not Can1, is required for growth of *C. neoformans* in its natural environment. *CAN1* and *CAN2* were disrupted in the *C. neoformans* strain KN99 (*MATα*), using the nourseothricin resistance gene (*NAT1*) as a marker. Two independent *can2*/*NAT1* mutants (NE417 and NE418) grew at 5% CO₂ but failed to grow in air (Fig. 7). Reintroduction of a single copy of *C. neoformans* *CAN2*, yielding NE425 (*can2*/*NAT1 HYG1::CAN2), restored the ability to grow and synthesize the capsule in air (Fig. 7 and data not shown, respectively). When grown in 5% CO₂, the *can2*/*NAT1* mutants displayed a nor-

**FIG. 6.** Growth inhibition of *C. neoformans* by ethoxyzolamide. Tenfold serial dilutions of *C. neoformans* cells were spotted (10⁵ to 10 cells) on YPD agar containing 4% dimethyl sulfoxide (control) or 3 mM ethoxyzolamide dissolved in 4% dimethyl sulfoxide and grown in 0.033% or 5% CO₂.

**FIG. 7.** Disruption of *CAN2* by biolistics, using the nourseothricin resistance gene as a marker. *C. neoformans* strain KN99 (*MATα*) was transformed by biolistics. The wild-type strain (NE241 [*MATα*]), two *can2* deletion mutants (NE417 and NE418 [*MATα* *can2*/*NAT1*]), and one revertant (NE425 [*MATα* *can2*/*NAT1 HYG1::CAN2*]) were screened for the ability to grow on YPD medium (pH 6.7 and 7.4) at 37°C in air or a physiological concentration of CO₂ (0.033% and 5%, respectively).
mal capsule like that of the wild-type strain NE241 (data not shown). In contrast, disruption of CAN1 did not affect growth or capsule biosynthesis when mutants were incubated in air or 5% CO2 (Fig. 7 and data not shown, respectively). This result was consistent with the low expression levels of CAN1, confirming that Can2 is the major CA in C. neoformans. Interestingly, Bahn et al. (5) reported that some fatty acids restored the growth of can2/H9004 mutants constructed in rich, but not synthetic, medium. This result suggests that the lack of bicarbonate production by can2/H9004 mutants affects the synthesis of fatty acids, causing a growth defect.

These data demonstrate that Can2 is only essential for C. neoformans growth under limiting concentrations of CO2. In this regard, this basidiomycete is similar to the ascomycete C. albicans, which requires CA for pathogenic growth in niches where low levels of CO2 are encountered (14). The observation that CA is not required for growth or capsule biosynthesis in the presence of physiological concentrations of CO2 implies that, when present at high levels, CO2 is spontaneously converted to HCO3\(^-\) to support growth and induce capsule biosynthesis. Interestingly, both CAN1 and CAN2 have been shown to be dispensable for the virulence of C. neoformans at the high concentrations of CO2 found during systemic infection (5). The dependence of C. neoformans on a CA to survive in its natural habitat reveals a requirement for CO2 equilibration with bicarbonate and suggests the existence of a bicarbonate-dependent signaling cascade.

**CO2-chemosensing activity of adenylyl cyclase is maximal at physiological pH.** C. neoformans capsule biosynthesis is dependent on the synthesis of cAMP by the Cac1 adenylyl cyclase. A truncated form of Cac1 containing the putative catalytic domain of the enzyme was cloned and expressed under the constitutive strong promoter TEF2 of C. albicans in the CR276 strain, which fails to form filaments in either air or elevated concentrations of CO2 (Fig. 8). Cac1 restored the capability of a C. albicans AC double mutant to sense CO2 and to form filaments, demonstrating that the region encoding amino acids 1825 to 2271 is sufficient to respond to physiological concentrations of CO2 and induce CO2-mediated morphology switching in C. albicans.

Moreover, consistent with our previous report (14), purified C. neoformans Cac1 was stimulated more than sixfold by bicarbonate, giving a 50% effective concentration of 4.3 ± 0.15 mM HCO3\(^-\) (Fig. 9A). The Henderson-Hasselbalch equation.

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**FIG. 8.** Restoration of CO2-dependent filamentation of C. albicans by a truncated form of C. neoformans adenylyl cyclase Cac1. The fragment encoding amino acids 1825 to 2271 of the C. neoformans adenylyl cyclase gene CAC1 was expressed in C. albicans (strain CR276) grown in Dulbecco’s modified Eagle’s medium, pH 7, in 0.033% or 5% CO2 at 37°C for 24 h. Cells were photographed at a magnification of ×70. Strain CR276 of C. albicans harboring pFM-2 was used a control.
predicts that in the presence of 5% CO$_2$ at physiological pH, the intracellular bicarbonate concentration will equilibrate to $\sim$25 mM HCO$_3^-$ in the absence of carbonic anhydrase activity. At 25 mM bicarbonate, the *C. neoformans* AC would be maximally stimulated to generate cAMP and promote capsule biosynthesis, rendering the CA superfluous. This dose-response relationship explains why CAN2 is dispensable when *C. neoformans* encounters elevated concentrations of CO$_2$, where sufficient amounts of HCO$_3^-$ are generated spontaneously to maintain cyclase activity. 

In order to address the significance of these results in the context of physiological conditions, the activity of Cac1 was assayed over a range of pHs. We demonstrate that bicarbonate stimulation of the *C. neoformans* AC is maximal at physiological pH and, similar to the basal cyclase activity, drops dramatically above pH 7.5 (Fig. 9B). This result elucidates why the capsule size increases at physiological pH, although an alkaline pH alone is not sufficient to regulate capsule size (37).

**CO$_2$ sensing in *C. neoformans*.** Taken together, these results allow us to propose a model for CO$_2$ sensing in *C. neoformans* (Fig. 10). CO$_2$ diffuses into the cell and is hydrated to bicarbonate by Can2 when present in limiting concentrations. HCO$_3^-$ stimulates Cac1 activity, resulting in the activation of the cAMP-signaling pathway, which controls major virulence determinants such as capsule biosynthesis. Can2 activity is dispensable when *C. neoformans* encounters elevated concentrations of CO$_2$, where sufficient amounts of HCO$_3^-$ are generated spontaneously to maintain cyclase activity. The molecular characterization of CO$_2$ sensing in *C. neoformans* provides a novel insight into microbial pathogenesis and may lead to the development of drugs interfering with the CO$_2$ signaling pathway.

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