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CO₂ Acts as a Signalling Molecule in Populations of the Fungal Pathogen Candida albicans

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Abstract

When colonising host-niches or non-animatated medical devices, individual cells of the fungal pathogen Candida albicans expand into significant biomasses. Here we show that within such biomasses, fungal metabolically generated CO₂ acts as a communication molecule promoting the switch from yeast to filamentous growth essential for C. albicans pathology. We find that CO₂-mediated intra-colony signalling involves the adenylyl cyclase protein (Cyr1p), a multi-sensor recently found to coordinate fungal responses to serum and bacterial peptidoglycan. We further identify Lys 1373 as essential for CO₂/bicarbonate regulation of Cyr1p. Disruption of the CO₂/bicarbonate receptor-site interferes selectively with C. albicans filamentation within fungal biomasses. Comparisons between the Drosophila melanogaster infection model and the mouse model of disseminated candidiasis, suggest that metabolic CO₂ sensing may be important for initial colonisation and epithelial invasion. Our results reveal the existence of a gaseous Candida signalling pathway and its molecular mechanism and provide insights into an evolutionary conserved CO₂-signalling system.

Introduction

Candida albicans is the predominant fungal pathogen of humans. In healthy individuals C. albicans resides as a commensal of the gastrointestinal, oral and vaginal tracts. C. albicans can cause superficial infections which, although not life threatening, provide discomfort to the individual and require treatment with antifungals which is a constant drain on hospitals resources. However, C. albicans infections are life threatening when the individual's immune system becomes compromised as a result of age, cancer, chemotherapy hospitalisation and AIDS. Under these circumstances superficial infections may readily develop into systemic disease where mortality rates are reported to be up to 40%, which is higher than those for most bacterial infections [1,2,3]. For example, oropharyngeal candidiasis is common in patients with haematological malignancies (up to 60%) and those undergoing radiotherapy [4,5,6]. Here, a few fungal cells develop into biomasses measuring several millimetres in diameter that penetrate and invade the underlying tissue, eventually leading to dissemination of Candida into the blood stream and subsequently systemic infection [7].

Development from superficial infection to invasive disease is mediated by many well characterised virulence factors including morphological transition. C. albicans can exist in yeast, pseudohyphal and true hyphal growth forms, all of which are important for the virulence of the organism [8]. Yeast cells are thought to be essential for growth and dissemination [9], while the hyphal forms are essential for invading mucosal membranes [9]. This morphological transition is mediated by host environmental cues including temperature, pH, serum, O₂, and CO₂, which the pathogen encounters during disease progression [5,10,11].

The virulence-associated morphological transitions of C. albicans are largely controlled through the secondary messenger cAMP. In C. albicans, cAMP is synthesised by the fungal adenylyl cyclase (AC), Cyr1p [12]. Among the Class III nucleotidyl cyclase family [13]. Activity of Cyr1p governs most processes essential to Candida virulence including tissue adhesion followed by the invasion of the underlying host-barriers, and biofilm formation [14]. Activity of Cyr1p is essential for C. albicans virulence and the production of the matrix molecules responsible for adherence to host surfaces. Inactivation of Cyr1p leads to a severe reduction in virulence in a disseminated candidiasis model of infection [15]. Cyr1p is also a key regulatory protein when colonising host niches or non-animatated medical devices. Here we show that within such biomasses, fungal metabolically generated CO₂ acts as a communication molecule for regulating Cyr1p activity within the colony.

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Author Summary

Pathogenic microorganisms can produce a variety of secondary metabolites and signalling molecules which can affect the host, or provide them with a selective advantage against competing commensal organisms. We demonstrate that gaseous, metabolically generated CO$_2$ can serve as a signalling molecule to enhance the organism’s virulence during infection establishment by using the fungal pathogen *Candida albicans* as a model. Furthermore, we identified a CO$_2$ receptor site within the catalytic domain of the soluble adenylyl cyclase, Cyr1p, which is critical for CO$_2$ sensing and hence virulence of the organism. CO$_2$ sensing is conserved in a variety of pathogenic species, and increased levels have been shown to suppress the host’s immune system. Thus, CO$_2$ sensing may represent a mechanism to enhance *C. albicans* virulence when the host’s immune system is suppressed.

In addition to host environmental cues, the morphological transition of *C. albicans* is also regulated by soluble chemical mediators, termed quorum sensing molecules (QSMs). QSMs are secreted into the environment by a variety of microorganisms ([for recent reviews see 17,18], and upon reaching threshold concentrations, impact on microbial behaviour by influencing expression of virulence determinants [19]. QSMs including the self-generated sesquiterpene farnesol [20] and 3-oxo-C12 homoserine lactone (HSL) secreted by *Pseudomonas aeruginosa* [21] inhibit *C. albicans* filamentation though cAMP dependent signalling cascades [22].

Further to soluble chemical mediators, volatile compounds can also act as signalling molecules. For example, in *Saccharomyces cerevisiae*, nutrient limited yeast cells release volatile ammonia, which when sensed by another colony inhibits its growth in the direction of the signal [23]. CO$_2$ is a volatile gas that has recently been described as a predominant regulator of *C. albicans* virulence factors and has been shown to effect the virulence of other microbial species [24,25]. In *C. albicans* CO$_2$ functions in two processes key to pathogenicity, one metabolic and the other cell signalling to promote filamentation [10]. In biological systems CO$_2$ is maintained in equilibrium with its hydrated form, HCO$_3^-$, via the actions of carbonic anhydrase. HCO$_3^-$ is required for metabolism, but when at high concentrations HCO$_3^-$ directly activates adenylyl cyclase increasing cytosolic cAMP and promoting filamentation [10]. To date, only the effects of high (5%) exogenous CO$_2$ concentrations have been investigated in microbial species. However, microbes continuously secrete metabolically generated CO$_2$ into their immediate microenvironment at levels perceived to be lower than 5%. Here, we investigate the effects of self generated CO$_2$ on pathogenicity associated traits of *C. albicans*. Previously we identified the carbonic anhydrase, Nce103p, as being essential for growth under CO$_2$ limiting conditions [10]. Now we explore a new application of the mutant strain Δnce103 as a CO$_2$ biosensor to report on CO$_2$ concentrations within fungal biomass. Using our CO$_2$-dependent biosensing strain, we demonstrate that build-up of self-generated, metabolic CO$_2$ occurs in a fungal population. Furthermore, we show that CO$_2$ mediates its effects as a hierarchy, with low concentrations of CO$_2$ functioning to fill metabolic demand, then once CO$_2$ exceeds a critical threshold, it promotes filamentation and subsequent surface invasion of the pathogen. We show that microbial CO$_2$, like environmental CO$_2$, is sensed by the AC catalytic domain and identify a bicarbonate receptor site in Cyr1p.

Results

*C. albicans*-generated CO$_2$ accumulates under diffusion-limiting conditions

CO$_2$ is generated during metabolism and acts as an important cellular signalling molecule in many organisms. CO$_2$ influences microbial virulence and organisms behaviours such as mating, feeding or ventilation [26]. We confirmed that, when grown under diffusion-limiting conditions (i.e., closed systems), *C. albicans* accumulated self-generated CO$_2$ (Figure 1 A, B). Next we asked whether self-generated CO$_2$ could be utilized by *C. albicans* to meet the organism’s growth requirements. In normal atmospheres, (0.03% CO$_2$) the *C. albicans* carbonic anhydrase (CA), Nce103p, is essential for catalyzing the hydration of CO$_2$ to bicarbonate to meet metabolic demands. Therefore, in ‘open’ systems (i.e., under the 0.03% CO$_2$ in air), deletion of *NCE103* results in a depletion of bicarbonate levels which inhibits growth. However, at elevated CO$_2$ concentrations (such as 5% CO$_2$ experienced by *C. albicans* when inside an infected mammalian host) there is sufficient CO$_2$ spontaneously hydrated to bicarbonate to meet the metabolic needs of the organism.

Figure 1. Closed systems enable CO$_2$ accumulation. 10,000 Wild type cells were inoculated onto 10 ml DMEM pH7 agar in BacT/ALERT bottles. Bottles were either incubated as an open system (A) where free diffusion of metabolically generated gases was permitted or as a closed system (B) where diffusion was inhibited. CO$_2$ accumulation was measured for 48 hr at 37°C in BacT/ALERT 3D automated microbial detection system (bioMerieux). Reflective units depicted on the X-axis are a direct measurement of the CO$_2$ concentration in the system (see material and methods for details). doi:10.1371/journal.ppat.1001193.g001
requirements restoring growth (Figure 2A). Therefore, the carbonic anhydrase mutant (TK1; Anel103) can only grow in environments with elevated concentrations of CO\(_2\) [10], and as a result, functions as a CO\(_2\) bio-indicator. The Anel103 bio-indicator strain failed to grow when co-incubated with wild type (SC5314; WT) cells in an open system, but grew in the presence of WT C. albicans in a closed system without exogenously supplied CO\(_2\) (Figure 2B). Furthermore, incubation of surplus (10,000 CFUs/plate) Anel103, on its own, in closed but not open systems also restored the growth of Anel103 (Figure S1), suggesting that in closed systems the elevated CO\(_2\) levels are sufficient to complement the growth of Anel103.

To confirm that it was volatile CO\(_2\) generated by the WT strain which restored growth to the Anel103 CO\(_2\) bio-indicator strain, we included hydroxide into the closed system, which specifically traps CO\(_2\) in the form of carbonate [27]. Solid sodium hydroxide interfered with the growth of the Anel103 CO\(_2\) bio-indicator strain, but not WT (Figure 2C). The diminished growth in the presence of sodium hydroxide is most likely caused by CO\(_2\) trapping and not oxygen depletion, as oxygen levels are not influenced by the CO\(_2\) trap. Taken together, these results reveal that metabolically generated CO\(_2\) can provide sufficient HCO\(_3^-\) to meet the metabolic demands of C. albicans, and that this CO\(_2\) can be provided in the form of a volatile signal from neighbouring colonies.

Consistent with the idea that the CO\(_2\) generated by WT C. albicans is supplying CO\(_2\)/HCO\(_3^-\) to meet the metabolic demand of the Anel103 bio-indicator strain, rescue was independent from cAMP signalling system, as the Cyr1-Anel103 strain (RH12) was also complemented when incubated at elevated CO\(_2\) (Figure 2A). These data suggest that there is sufficient CO\(_2\) generated during normal metabolism of WT C. albicans to support the growth of the Anel103 bio-indicator strain, as long as diffusion of the generated CO\(_2\) is limited.

CO\(_2\) accumulates inside a fungal biomass

We next asked whether CO\(_2\) levels sufficient for signalling would build-up within a fungal biomass. To address this question, we grew the Anel103 CO\(_2\) bio-indicator strain on its own, or mixed with equal numbers of DAY286, a Abir1 strain which is wild type for carboxic anhydrase and adenylyl cyclase, in an open system to specifically test whether CO\(_2\) accumulation could occur between cells growing in the same biomass. Using the different auxotrophic tags (HIS\(^+\) and HIS\(^-\)) to distinguish the two strains after incubation within mixed biomasses, we were able to directly test whether metabolically generated CO\(_2\) from DAY286 could complement the growth of Anel103, while Anel103 on its own would be restricted in growth. Co-incubation of Anel103 with DAY286 enhanced the recovery of Anel103 600-fold (p = >0.0001) when compared to incubation of the CO\(_2\) bio-indicator strain on its own (Figure 3). To exclude that DAY286 was able to fill the metabolic demands of Anel103 by providing other metabolic intermediates other than CO\(_2\), the Anel103 strain was also co-incubated with a surplus (1×10⁶ cells) of heat-killed DAY286 cells. However, co-incubation of Anel103 and heat-killed DAY286 did not enhance the recovery of Anel103 compared to incubation of the CO\(_2\) bio-indicator strain alone (Figure 3, p = >0.0001), suggesting that within a fungal biomass, even in an open system, there is an accumulation of metabolic CO\(_2\) sufficient to promote the growth of Anel103. These data also prove that the carbonic anhydrase is essential because it ‘captures’ metabolically generated CO\(_2\) as HCO\(_3^-\) which is needed to meet metabolic requirements of cells deep within the colony.

The volatile messenger CO\(_2\) affects C. albicans colony morphology

CO\(_2\) is not only required for metabolism, but it also acts as a signal for cAMP-dependent filamentation of C. albicans. Therefore, we sought to determine whether metabolically generated CO\(_2\)
Furthermore, microscopic inspection of WT colonies, after co-plating WT and nce103, revealed that nce103 colonies formed wrinkled colonies (Figure 4D), which were not biomass dependent. At 48 hours, plating 500 CFUs produced extended filamentous colonies (Figure 4A). Microscopic analysis of resuspended colonies separated by plating onto selective media and nce103 CFUs counted. Values are the mean and standard deviation from 8 independent experiments (** indicates that the P statistic for the represented data was greater than 0.0001). doi:10.1371/journal.ppat.1001193.g003

could act within a biomass to modulate morphology. To test whether *C. albicans* produces sufficient CO2 to affect filamentation, we incubated wild type cells under open and closed conditions. In open systems, no colonies filamented, while under closed conditions, we observed changes in colony morphology after 48 hours. Plating 500 CFUs produced extensive filamentous colonies (Figure 4A). Microscopic analysis of resuspended colonies confirmed that the majority of the cells filamented in the closed system (forming a highly interwoven mass of cells resistant to mechanical stress; Figure 4A). As *C. albicans* hyphal induction is critically dependent on cAMP signalling cascades [12], we tested a strain deficient for adenyl cyclase CR276-CTRL (RH20; Δcyrl) in our open and closed systems. The Δcyrl strain did not show any changes in colony morphology, even when incubation periods were extended to 72 hours to account for its known reduced growth rate (Figure 4B). Additionally, altered morphology was independent of carbonic anhydrase, as Δnce103, but not Δcyrl, formed filaments in the presence of 5% CO2 (Figure 4C). Interestingly, the extent of filamentation of the WT strain was biomass dependent. At 48 hours, plating in the presence of 50 CFUs generated wrinkled colonies (Figure 4D), which were not fully filamentous.

Addition of the hydroxide trap into the closed system inhibited the morphological transition observed previously (Figure 4E). Furthermore, microscopic inspection of WT colonies, after co-incubation with Δnce103 in closed environments, confirmed that the colonies were smooth, round, yeast colonies, similar to those observed in the open system (data not shown). These observations suggest that the Δnce103 strain acts as a CO2 sink removing the majority of the gas from the system. Therefore, *C. albicans* produces CO2 which affects morphology, and cAMP is essential for the observed morphological effects.

**Fungal CO2/HCO3− sensing is mediated by lysine 1373 of the Cyr1p catalytic domain**

Directly testing the in vivo relevance of CO2 chemosensing would be greatly facilitated by an adenyl cyclase variant with specifically diminished CO2 sensitivity. Previously we have shown that CO2/HCO3− activates the catalytic domain of the fungal adenyl cyclase, Cyr1p [10], confirming that this Class III AC belongs to the bicarbonate-responsive soluble AC (sAC) subfamily [13,28]. Structural studies and *in vitro* work on mutated bacterial sAC-family enzymes indicated a mechanism for bicarbonate regulation, along with a potential bicarbonate binding site [29,30]. However, it remains to be shown whether the mechanism of activation and potential binding site generally apply to sAC-like enzymes, in particular from eukaryotes, and whether they are responsible for the *in vivo* effects of CO2 on AC activity.

Using sequence alignments of Class III ACs, we generated a homology model of Cyr1p and identified the Cyr1p site corresponding to the proposed bacterial CO2 receptor site (Figure 5A) [13,29]. A lysine residue [29], Lys1373 in *C. albicans* Cyr1p, would be a key interaction partner for bicarbonate in this receptor site. Class III ACs are dimers with shared active sites – i.e., residues from both monomers contribute to each active site – so that only the dimer can display activity. In contrast to ‘heterodimeric’ Class III ACs, which have one active site and a second, related-but-degenerated, ‘regulatory’ site in their dimer interface, homodimeric Class III ACs, like Cyr1p, have two identical catalytic sites in their dimer interface. In these ACs, it is believed that both sites can act as active or as regulatory sites. The putative bicarbonate-interacting lysine residue is strictly conserved in both ‘active’ and ‘regulatory’ sites (for example, in mammalian sAC, Lys334, would be the corresponding residue in the active site). In active sites, the conserved lysine at this position is essential for substrate binding [13]. Because Lys1373 of Cyr1p should be essential for substrate binding in at least one of the two sites formed at the homodimer interface, we predicted *Cyr1*ΔLys1373 would be inactive on its own. We integrated full-length Cyr1p with Lys1373 point mutated to alanine, under the control of the TEFP promoter, into an adenyl cyclase null, generating strain CR276-CYR1ΔLys1373 (RH22; cyrl/DYF2 Cyr1ΔLys1373). CR276-CYR1ΔLys1373 was refractory to both CO2 and serum induction of filamentation, behaving similarly to the vector-control strain CR276-CTRL (RH20; Figure S2). Thus, *Cyr1*ΔLys1373 homodimers encode a non-functional AC.

To specifically test the role of this lysine in the bicarbonate activation of Cyr1p and to generate an AC with a selective defect in its bicarbonate responsiveness, we generated strains containing mutant/WT heterodimers. Due to the dimeric architecture of Class III ACs, one wild type Cyr1p monomer could interact with one Cyr1pΔLys1373 monomer, allowing basal AC activity, but preventing bicarbonate stimulation due to disruption of the bicarbonate interacting site in the second ‘regulatory’ centre (as described above). The point mutated Cyr1p was integrated into a strain expressing wild type adenyl cyclase, generating strain CA14-CYR1ΔLys1373 (RH25; *Cyr1/Cyr1*ΔLys1373). Consistent with the expected heterodimer formation and with specific interruption of CO2-induced cAMP formation, CA14-CYR1ΔLys1373, but not the control strain CA14-CYR1 (RH24; *Cyr1/Cyr1*ΔLys1373), displayed a signal-specific defect to the filamentation inducing cues (Figure 5B, C), despite the two strains expressing comparable levels of *Cyr1*, (Figure S3). CA14-
CYR1\textsubscript{1373} filamented in response to serum, but much less in response to CO\textsubscript{2} while the control strain expressing wild type AC, CAI4-CYR1, filamented equally in response to both serum and CO\textsubscript{2}. The incomplete suppression of CO\textsubscript{2}-induced filamentation in CAI4-CYR1\textsubscript{1373} is consistent with the statistical formation of homodimers and heterodimers between the co-expressed wild type and variant protein, which will also yield fully CO\textsubscript{2}-sensitive wild type homodimers.

To confirm that the observed phenotype is specific to the destruction of the bicarbonate binding site in the \textit{CYR1}\textsubscript{1373} heterodimers, rather than a general influence on AC activity, two additional point mutations, that inactivate Cyr1p stimulus-independent, were constructed. Asp1334 and Asp1377 (involved in active site Mg\textsuperscript{2+} binding) were mutated to Ala and also expressed under the control of the \textit{TEP2} promoter. Integration of these constructs into the adenyl cyclase mutant (CR272-CYR1\textsubscript{1373}; RH26, and CR276-CYR1\textsubscript{1373}; RH27) confirmed that the proteins were catalytically inactive (Figure S2). However, expression of these inactive proteins in CAI4 (containing two genomic copies of \textit{CYR1}; CAI4-CYR1\textsubscript{1373}; RH28, and CAI4-CYR1\textsubscript{1377}; RH29) did not perturb hyphal induction in response to 5% serum or elevated CO\textsubscript{2}, as Lys1373 did (Figure 5B, C). Thus, CAI4-CYR1\textsubscript{1373} shows a specific disruption of CO\textsubscript{2}-induced filamentation and therefore, the identified lysine, which likely acts as bicarbonate binding site, serves as physiological CO\textsubscript{2} “switch” in Cyr1p and perhaps in all related sAC-type enzymes.

Self-generated CO\textsubscript{2} is the volatile signal that accumulates under diffusion-limiting conditions and induces filamentation

We next directly tested whether CO\textsubscript{2} is the volatile messenger inducing \textit{Candida} filamentation by taking advantage of the CO\textsubscript{2} insensitive mutant strain CAI4-CYR1\textsubscript{1373}. CAI4-CYR1\textsubscript{1373} showed an attenuated response in the closed system, with 30% (±9%, P = 0.001) of cells producing smooth colonies indicative of reduced filamentation, while CAI4-CYR1, CAI4-CYR1\textsubscript{1374} and CAI4-CYR1\textsubscript{1373} were 100% filamentous (Figure 5C). However, CAI4-CYR1\textsubscript{1373} cells that were inoculated onto DMEM agar supplemented with 5% serum always produced 100% filamentous colonies, confirming that the reduced filamentation was signal-specific (Figure 5B, C); i.e., CO\textsubscript{2}-induced differentiation was diminished while serum-induced differentiation was unaffected.

CO\textsubscript{2} activation of Cyr1p may have a role in pathogenicity

The morphological transition of \textit{C. albicans} is essential to the organism’s virulence. As CO\textsubscript{2} is a potent inducer of hyphal development we tested whether the identified CO\textsubscript{2}-recognition-mechanism regulates \textit{C. albicans} pathogenicity in an \textit{in vivo} model. Initially to test this hypothesis, we selected the Toll-deficient \textit{Drosophila melanogaster} infection model to provide a controlled yet reduced (in respects to filament inducing cues) environment, as only a subpopulation of the cells were CO\textsubscript{2} insensitive. \textit{D. melanogaster} is a potent inducer of hyphal development we tested whether the identified CO\textsubscript{2}-recognition-mechanism regulates \textit{C. albicans} pathogenicity in an \textit{in vivo} model. Initially to test this hypothesis, we selected the Toll-deficient \textit{Drosophila melanogaster} infection model to provide a controlled yet reduced (in respects to filament inducing cues) environment, as only a subpopulation of the cells were CO\textsubscript{2} insensitive. \textit{D. melanogaster}
melanogaster was infected with CAI4-CYR1\textsuperscript{1373} and CAI4-CYR1 and survival assessed over 48 hours. Although the percentage mortalities of Toll-deficient D. melanogaster infected with either strain were similar at the end-point of the time-course experiment, CAI4-CYR1\textsuperscript{1373} killed D. melanogaster at a significantly slower rate (p = 0.005) compared to CAI4-CYR1 (Figure 6A). The reduced virulence of CAI4-CYR1\textsuperscript{1373} over CAI4-CYR1 was not attributed to differences in growth rates or fungal burden, as these were comparable between the two strains (Figure S4 and Table S1).

To investigate how the point-mutated adenyl cyclase would affect the virulence of C. albicans in the mammalian host, the mouse model of disseminated candidiasis was utilised. CAI4-CYR1 and CAI4-CYR1\textsuperscript{1373} displayed no significant difference in their ability to cause system infection after intravenous injection (Figure 6B). There was, however, a greater degree of variation in fungal burdens, weight loss and outcome scores compared with control strain (Table S2), which may be reflective of the different populations obtained in the CAI4-CYR1\textsuperscript{1373} strain (i.e. 70% CO\textsubscript{2} responsive and 30% CO\textsubscript{2} non responsive).

As the fly model identified that CYR1\textsuperscript{1373} was delayed in its ability to cause infection, we also sampled mice at days 1, 2 and 3 post-infection to determine whether the delayed ability of CYR1\textsuperscript{1373} to cause infection was also present in the mouse model. However, there were no statistically significant differences in kidney burdens, weight changes or outcome scores, but again there

**Figure 5. Mechanism of CO\textsubscript{2} sensing in C. albicans.** A) Homology model of the Cyr1p homodimer with bound substrate analogue and the activator bicarbonate. The protein chains are shown in green and cyan, respectively, and ligands and functional residues mutated in our study are shown in stick representation and labelled with residue number and chain A or B. Lys\textsubscript{1373} can either bind the substrate base (active site) or bicarbonate (regulatory site). B) Point-mutated CYR1 (1373, 1334 and 1377 aa), and control plasmids (wt CYR1 and the vector control pSM2) were integrated into in\textit{URA3} locus of CAI4. Colonies were grown on DMEM pH7 itself, or supplemented with either 5% serum, or 5% CO\textsubscript{2}. The colony depicted for CAI4-CYR1\textsuperscript{1373} displaying an attenuated response to CO\textsubscript{2} is representative of approximately 30% of the population. The differential response to CO\textsubscript{2} is hypothesised to result from the variations in levels of CO\textsubscript{2}-responsive Cyr1p homodimers, Cyr1-Cyr1\textsuperscript{1373} heterodimers and non-functional Cyr1p\textsuperscript{1373} homodimers. All other colonies are representative of the entire population (scale bar represents 100 \textmu m). C) Cell suspensions of the desired strains were inoculated onto DMEM pH7 supplemented with 10% serum or CBA agar (CBA plates were either incubated in open or closed systems) and after 48 hours colony morphology assessed. Results are the mean and standard deviations from 4 replicate experiments (** indicates that the P statistic for the represented data is greater than 0.001).

**Figure 6. CAI4-CYR1\textsuperscript{1373} may have implications for virulence.** A) Toll deficient D. melanogaster were infected by injection into the thorax with C. albicans strains CAI4-CYR1, CAI4-CYR1\textsuperscript{1373} or sterile YDP (CTRL). Experiments were performed with groups of 15 adult flies and incubated at 30 °C for 38 hours. Values represent the mean and standard deviation from 5 independent experiments. B) CAI4-CYR1, CAI4-CYR1\textsuperscript{1373} were intravenously injected (2.4–2.5 \times 10\textsuperscript{4} CFU/g) into 6 female BALB/c mice (6–8 weeks old) and survival monitored. Mice were culled when they showed signs of severe illness or their weight had decreased by more than 20%.

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was greater variability in the CAH4-CYR1\textsuperscript{1373} data, which was not observed for the CAH4-CYR1 strain (Table S2). The differences in outcome between the two infection models may be expected. Although the CAH4-CYR1\textsuperscript{1373} strain is reduced in its ability to filament in response to elevated CO\textsubscript{2}, it is responsive to serum or elevated temperature, cues absent in the fly model.

**Discussion**

CO\textsubscript{2} is a biologically important molecule and has major implications for disease progression. As well as host derived CO\textsubscript{2}, microorganisms themselves generate and secrete metabolic CO\textsubscript{2} into their microenvironment which has the potential to impact on the organism’s virulence. We observed that fungal derived, metabolic CO\textsubscript{2} accumulated in *C. albicans* biomasses to sufficient levels to first provide HCO\textsubscript{3}\textsuperscript{-} as a metabolic intermediate to promote growth and then subsequently to induce the morphological transition crucial for *C. albicans* pathogenicity through activation of Cyr1p via lysine residue 1373.

CO\textsubscript{2} is produced by multiple metabolic processes and the data presented here suggest that nutrient availability affects production rates. For instance, we found that fungal biomasses grown on nutrient rich media (YPD) were able to support the growth of over ten times the amount of our bio-indicator strain (*Ansec103*) compared to those grown on nutrient limiting media (YNB; data not shown). This result may reflect the increased flux through metabolic pathways as the organism utilises the available nutrients. In accordance with this Ghosh *et al.* recently proposed that the catalysis of arginine to urea and urea’s subsequent breakdown to CO\textsubscript{2} produces sufficient CO\textsubscript{2} to induce *C. albicans* germ tube formation when engulfed by macrophages [31]. Therefore, arginine biosynthesis maybe a key contributor to CO\textsubscript{2} production in *C. albicans*.

Accumulation of metabolically generated CO\textsubscript{2} in race tubes has been shown to impact on asexual spore development in *Neurospora crassa* [32,33]. Here, simple displacement of the accumulated CO\textsubscript{2} (by inverting the tubes) restores conidial handing. These results suggest that the heavier density of CO\textsubscript{2} compared to O\textsubscript{2} and N\textsubscript{2} allow it to accumulate in a system more freely rather than diffusing away. In accordance with this, we found that growth of the *Ansec103* strain was enhanced at the bottom of the colony (17-fold, *P* = 0.001) where agar invasion was observed to stem from the centre of the colony, suggesting that the concentration of CO\textsubscript{2} is highest at the lower extremities of the biomass (data not shown).

The ability to accumulate in a system is essential for communication molecules, with many molecules only having an impact once a threshold concentration is reached. However, unlike conventional QSMs, CO\textsubscript{2} may not be specifically generated for the purpose of communication. This is mainly due to the lack of evidence for a single pathway controlling CO\textsubscript{2} output, although the work of Ghosh *et al.* suggest that arginine biosynthesis may play a significant role in the production of CO\textsubscript{2} in *C. albicans* [31]. Therefore, it is more likely that the organisms have evolved to sense and respond to CO\textsubscript{2} gradients as a form of diffusion sensing rather than CO\textsubscript{2} being a true quorum sensing molecule.

However, the interplay between CO\textsubscript{2} production and other microbial species maybe relevant. When colonising mucosal membranes and epithelia *C. albicans* will be in contact with other microbes residing in the same niche. For example we found that under diffusion limiting conditions significantly fewer colony forming units (10-fold less) of *Escherichia coli* or *Pseudomonas aeruginosa* were required to restore growth of the CO\textsubscript{2} bio-indicator strain, *Ansec103*, compared to wild type *C. albicans* (data not shown). Given that *C. albicans* is found in mixed microbial biofilms on medical devices it is interesting to speculate about the role the metabolically generated CO\textsubscript{2} in biofilm establishment and maintenance.

Signalling molecules normally interact with membrane associated receptors to initiate intracellular signalling cascades terminating in a transcriptional response which subsequently induces the desired effect. Unlike most signalling molecules, CO\textsubscript{2} enters the cell by simple diffusion and is maintained in the cell through hydration to HCO\textsubscript{3}\textsuperscript{-} via the actions of carbonic anhydrase. Although HCO\textsubscript{3}\textsuperscript{-} is a metabolic intermediate and will feed into various metabolic processes, a conserved HCO\textsubscript{3}\textsuperscript{-} binding site was identified in the adenyl cyclase, Cyr1p, involving lysine residue 1373, which enables CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-} to bind and directly stimulate Cyr1p and hence activate cAMP dependent signalling cascades. Mutation of the HCO\textsubscript{3}\textsuperscript{-} binding site resulted in a subpopulation of cells that were CO\textsubscript{2} non responsive.

Introduction of the CO\textsubscript{2} sensing deficient strain (CAH4-CYR1\textsuperscript{1373}) into the Toll-deficient *D. melanogaster* infection model highlighted its reduced ability to kill the host. In the mouse model for disseminated candidiasis this attenuated virulence was not observed. However, this was hypothesised as the mutated strain remained fully responsive to other host environmental cues, including the elevated temperature and presence of serum in mammals, which are absent in the fly infection model. Taking this into consideration we hypothesise that the ability to sense and respond to metabolically generated CO\textsubscript{2} gradients is important during colonisation and initial invasion of mucosal membranes lining the oral and vaginal tracts during superficial infections where environmental CO\textsubscript{2} conditions are low and not as important during systemic infection (Figure 7). Here, in an expanding fungal biomass self produced metabolic CO\textsubscript{2} gradually accumulates and once reaching threshold concentrations directly activates the soluble adenyl cyclase, Cyr1p via the catalytic, bicarbonate receptor site. The resulting increase in cytosolic cAMP, in conjunction with other epithelial adhesion mechanisms, functions to induce the morphological switch in *C. albicans*. Hyphal formation results in the penetration and invasion of the underlying epithelial cells, which subsequently enhances the dissemination of the fungal pathogen. Our data supports this as we routinely found enhanced levels of *Ansec103* cells in the biomass sections that were invading into the agar, similar to what is observed in oropharyngeal candidiasis, suggesting that cells towards the bottom of the biomass are exposed to higher concentrations of CO\textsubscript{2} than cells on the surface, which would support hyphal development. Therefore, we hypothesise that during superficial infections that occur in niches where environmental CO\textsubscript{2} concentrations are low (for example, on the skin and mucosal membranes lining the oral cavity) *C. albicans* can use self generated, metabolic CO\textsubscript{2} to enhance adhesion and promote filamentation of the underlying cells increasing the opportunity for dissemination into the bloodstream.

In line with CO\textsubscript{2} playing an enhancing role in microbial virulence, hypercapnia (elevated CO\textsubscript{2}) has recently been shown to inhibit the production of anti-microbial peptides in *Drosophila* [34]. Furthermore, elevated CO\textsubscript{2} levels suppress the mammalian inflammatory response [35,36,37]. Therefore, pathogen associated, metabolically generated CO\textsubscript{2} may play multiple roles in the infection process. One would operate at a local level, suppressing the host’s immune system in the underlying epithelia and rendering the host susceptible to infection. Secondly, high CO\textsubscript{2} would enhance the microbe’s pathogenicity, providing more opportunity for host cell invasion.

In conclusion, Cyr1p is a multifunctional sensor that is essential to fungal pathogenicity. It contains multiple domains that mediate
signal-specific enzyme activation in C. albicans in response to diverse filamentation-inducing molecules. We have now identified the mechanism by which this AC is stimulated in vitro and in vivo by CO\textsubscript{2} supplied by the environment or the fungal biomass itself. Our results give novel molecular insights into this pathogenicity mechanism, as well as an evolutionary conserved CO\textsubscript{2}-chemoreception system. Interfering with fungal CO\textsubscript{2}-sensing may reveal novel approaches for therapeutic intervention.

**Methods**

**Ethics statement**

All animal experimentation was done in accordance with United Kingdom Home Office regulations and was approved by both the Home Office and the University of Aberdeen ethical review committee. All mice were checked and weighed at least once daily, and if they showed any signs of severe disease and/or had lost 20% of their original body weight mice were humanely terminated immediately. Mice sampled at defined time points were also humanely terminated prior to aseptic removal of kidneys for burden determination.

**Strains and media**

*C. albicans* strains and transforming plasmids used in this study are listed in Table S3. Columbia blood agar plates (CBA), a quality-controlled growth medium routinely used in diagnostic microbiology laboratories, supplemented with 5% defibrinated horse blood were either purchased premade, or were made from Columbia blood agar base [38] from Oxoid (2.3% peptone, 0.1% starch, 0.5% NaCl, 1% agar, pH 7.3). Dulbecco’s Modified Eagle Medium (DMEM) without bicarbonate and pyruvate was obtained from Gibco and used at pH7, (1.34% DMEM, 3.57% HEPES supplemented to a final concentration of 2% glucose). YNB and YPD were made as described previously [10]. Where supplementation with 5% CO\textsubscript{2} was required, plates were incubated in a CO\textsubscript{2} incubator (Infors HT Minintron) enriched with 5% (vol/vol) CO\textsubscript{2}. Solidified or serum supplemented media contained 2% agar and 5% horse serum.

Toll transheterozygotes flies were generated by crossing flies carrying a loss of function allele of Toll (T\textsuperscript{u-13584}, obtained from the Tubingen Drosophila Stock Collection) and flies carrying a thermo-sensitive allele of Toll, with a strong phenotype at 29°C (T\textsuperscript{u}; obtained from the Bloomington Stock Center). All stocks were maintained on standard fly medium at 25°C, except during infection experiments where flies were incubated at 30°C.

**Open and closed systems**

For diffusion-permitting (open) systems, plates were incubated in the standard way with no additional sealing mechanism. To generate a diffusion-limiting (closed) environment, standard 10 cm petri dishes containing CBA (20 ml) were sealed with two layers of laboratory sealing film (Parafilm) followed by three layers of standard cling-film (low density polyvinyl chloride). To minimise diffusion the sealing process was repeated twice. When plates were to be incubated in parallel, standard petri dishes were placed into, zip-locked polyvinyl chloride bags (15.5×23 cm). To ensure that the bags were air tight they were sealed mechanically with an additional polyethylene bar making the bags both air and water tight.

**Measurement of CO\textsubscript{2} accumulation in diffusion-limiting conditions**

Sodium hydroxide was used as a CO\textsubscript{2}-trap as described by the equation below. Plates were incubated in air tight plastic bags containing a separate vial of 4M NaOH, or 0.5g of solid NaOH crystals for 48 hrs. To measure CO\textsubscript{2} accumulation the BacT/ALERT system [39] was used with some modifications. The prefilled bottles were emptied in a sterile environment, media replaced with 20 ml of solidified DMEM pH7 and the agar surface seeded with 10,000 SC5314 cells. Bottles for incubation in closed systems were sealed as described for agar plates. CO\textsubscript{2} accumulation was directly measured using a BacT/ALERT 3D automated microbial detection system (bioMerieux) where microbial CO\textsubscript{2} production is assessed by a colorimetric sensor and detection system (red L.E.D and red-light-absorbing photodiode). Emitted light is recorded as a voltage signal that is directly

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**Figure 7. Model for metabolic CO\textsubscript{2} signalling in fungal pathogenicity.** As the cells proliferate on the epithelial surface, increasing fungal growth generates pockets of elevated CO\textsubscript{2} located at the bottom of the biomass. Cells exposed to the elevated CO\textsubscript{2} undergo morphological switching, promoting hyphal development and hence increasing the adherence of the organism. At the same time, the protruding hyphae would expose the pathogen to environmental signals like serum, pH and further increases in CO\textsubscript{2} levels, which would further enhance hyphal development, increasing the opportunity for tissue invasion.

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proportional to the reflective light and hence the concentration of CO$_2$ in the bottle.

$$\text{CO}_2 + 2\text{NaOH} \rightarrow \text{Na}_2\text{CO}_3 + \text{H}_2\text{O}$$

**Heterogeneous populations of CAI4 and *Ace103***

Heterogeneous cell suspensions containing equal proportions (500 cells/μl) of DAY286 and *Ace103* were spotted (1 μl total) onto individual YPD or YNB plates and incubated at 37°C for 48 hrs. Initially 1 ml of sterile water was used to wash the single colony from the plate with light agitation of the agar to remove adhered cells. From the recovered 800 μl, 200 μl was plated onto YNB, 5% CO$_2$ to promote growth of the strictly CO$_2$-requiring strain *Ace103* strain only (DAY286 will not grow under these conditions as it is *His1*/*His3*). Stability of the different phenotypic markers was verified upon replica-plating of colonies. The number of colonies was counted, and after taking into account the dilution factor, related back to the initial number of colonies in the cell suspension. Initial cell suspensions were always replica plated onto YNB and YPD to obtain the average starting cell concentration for each strain.

**Molecular modelling of a nucleotide and bicarbonate complex of homodimeric Cyr1p catalytic domain***

The amino acid sequence of Cyr1p was duplicated and aligned with the sequences of two homodimeric substrate analogue complex of CyaC from *Spirulina platensis* [PDB ID 1WC0; [30]] by using Genedoc (http://www.psc.edu/biomed/genedoc). A homology model for Cyr1p was generated with this alignment using Modeller [40], and nucleotide and divalent ions positioned by superposition with the experimentally determined CyaC complex structure. Bicarbonate was then positioned manually at the sites of pSM2. The 5′ domain of CYR1 together with the sequences of two chains of a homodimeric substrate analogue complex of CyaC from *Spirulina platensis* was integrated into the pSM2 plasmid forming plasmid pSMTC. The model was visualized using Pymol (DeLano Scientific; http://www.pymol.org).

**Site directed mutagenesis of CYR1***

Lys1375, Asp1334 and Asp1377 were point mutated to Ala by site directed mutagenesis using the following sets of primers (mutations underlined) 1373F-gggatatgaagtg and Primer 7-ctattaagcttgctagttctag, Primer 8-aacttgttcagctcagctcag and 1373R-atcaggttcctaccaagtattctcctctcctinsertioncctatc, 1334F-ggttttcactccgcactcgt and Primer 7, 1373R-ccatgaacgc

**Supporting Information***

**Figure S1** *Ace103* can promote its own growth under diffusion limiting conditions. 10,000 CFUs of *Ace103* were plated onto CBA media and incubated in open or closed systems for 48 hr. Found at: doi:10.1371/journal.ppat.1001193.s001 (0.58 MB TIF)

**Figure S2** Mutations in *CYR1* affect adenylyl cyclase activity as homo, but not heterodimers (related to Figure 5). The desired point mutated adenylyl cyclase genes or control plasmids were integrated into the *UR13* locus of CR276 and CAH, generating strains RH20-25 (Figure 7A) using standard heat-shock procedures as previously described [41].
**Figure S3** Expression levels of *CYR1* constructs (related to Figure 5 and Figure 6). A) Schematic diagram of the *CYR1* locus and the CAI4 *URA3* locus containing the integrated cassette. B) Strains were checked for single copy integration of plasmids containing *CYR1* and *CYR1* analogues as analysed by semi quantitative RT-PCR. Values are the mean and standard deviation from two independent experiments.

**Figure S4** CAI4-*CYR1* and CAI4-*pSM2* have the same growth rates (related to Figure 6). Overnight cultures were diluted to an initial OD

**Table S1** Fungal burden in the *D. melanogaster* infection model (related to Figure 6A). Flies were homogenised in sterile water and CFUs determined on YPD agar supplemented with chloramphenicol.

**Table S2** Mouse infection parameters measured on day 1–3 post-infection (related to Figure 6B). For each *C. albicans* strain 9 mice were challenged intravenously, with three mice sampled on days 1, 2 and 3 post-infection.

**Table S3** Strains used in the study.

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**Author Contributions**

Conceived and designed the experiments: RAH DMM GKR YW CS FAM. Performed the experiments: RAHS DMM HT RE YW. Analyzed the data: RAH LDS JWB GKR LJB YW NARG CS FAM. Contributed reagents/materials/analysis tools: JWB YW NARG. Wrote the paper: RAH LDS DMM GKR LJB CS FAM.

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