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ORIGINAL ARTICLE

The bacterial signalling molecule indole attenuates the virulence of the fungal pathogen *Candida albicans*

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Abstract

Aims: Indole is a signalling molecule, produced by a number of Gram-positive and Gram-negative bacteria both in nature as well as clinical environments. Here, we explored the effect of bacterial indole and one of its main derivatives on the virulence of the fungal pathogen *Candida albicans*.

Methods and Results: We found that indole and its derivate indole-3-acetonitrile (IAN) did not affect the viability of *C. albicans*. Interestingly, indole and IAN repressed *C. albicans* biofilm formation as well as the attachment of *C. albicans* to intestinal epithelial HT-29 cells and inhibited the ability of the yeast to make filaments that are the main virulence factor of *C. albicans*. In addition, we used the heterologous model host *Caenorhabditis elegans* to demonstrate *in vivo* that the presence of indole or IAN attenuates *C. albicans* infection ($P = 0{\cdot}0188$ and $P < 0{\cdot}0001$ for indole and IAN, respectively, compared to worms exposed to *C. albicans* DAY185 alone) and decreases fungal colonization in the nematode gut. Importantly, quantitative real-time polymerase chain reaction (qRT-PCR) results showed that in *C. albicans*, indole and IAN strongly stimulated the transcription of *NRG1*.

Conclusions: Indole and IAN attenuates fungal virulence by regulating the transcription of *NRG1*, a transcriptional factor that influences filamentation and biofilm formation in *C. albicans*.

Significance and Impact of the Study: Our findings indicate that the bacterial signalling molecules indole and its derivatives play an inter-kingdom role in dynamic network of microbiota and directly modulate the virulence of fungal *C. albicans* via *NRG1*.

Introduction

In polymicrobial communities, bacteria and fungi directly and indirectly influence each other's growth, survival and physiology through multi-factorial pathways (Hogan and Kolter 2002). In particular, the virulence of bacteria or fungi can be influenced by substances produced by other microbes and secreted molecules mediate many types of interactions between bacteria and fungi (Peleg *et al.* 2010). However, despite the ubiquitousness of bacterial–fungal competition, little is known about the identities of these signalling molecules and their effects. In the microbe world, studies that investigate these interactions are particularly valuable, because a complex microbial community that involves a number of signalling networks is present in nature as well as in clinical environments including human intestinal tract (Hsiao *et al.* 2008; Lee and Lee 2010).

A number of studies demonstrated that extracellular signalling molecules from bacteria can mediate quorum sensing (QS) and QS molecules produced by one organism and can affect entire cell populations (Miller and Bassler 2001). Importantly, many bacterial genes, including virulence factors, are stimulated by these small signalling

molecules produced by individual cells of the same or different species (Peleg *et al.* 2010). Several species including *Pseudomonas aeruginosa* employ their own signalling molecules (autoinducer-1 or AI-1, a family of related homoserine lactones) for intraspecies communication purposes, whereas other bacterial species (e.g. *Escherichia coli* and *Salmonella* spp.) use a common signalling molecule, AI-2, for interspecies and universal communications (Surette and Bassler 1998; Rasmussen *et al.* 2005). However, there are a few reports on the cross-kingdom effects of the universal signalling molecules.

A variety of both Gram-positive and Gram-negative bacteria produce indole as an intracellular signalling molecule (Lee and Lee 2010). It has been reported that indole can control the virulence of pathogenic bacteria (Lee *et al.* 2011) as well as modulate the host immune system (Bansal *et al.* 2010). In addition, several indole derivates such as indole-3-acetonitrile (IAN), which is a plant-associated growth hormone originated from broccoli, cauliflower and cabbage, could inhibit the biofilm formation and virulence factor productions of *Ps. aeruginosa* and enterohaemorrhagic *E. coli* (EHEC) O157:H7 (Lee *et al.* 2011).

Similar to bacteria, the pathogenic fungus *Candida albicans* produces at least two signalling molecules called farnesol and tyrosol that influence growth, virulence and other physiological pathways (De Sordi and Muhlschlegel 2009). Although inter-kingdom signalling molecules play an important role in competition between bacteria and fungi, or the underlying molecular pathways involved (Hogan and Kolter 2002; Peleg *et al.* 2008), their roles in micro-organism world are still unclear. Here, we provide direct evidence that bacterial indole and IAN influence the virulence of fungal *C. albicans*, by altering the attachment as well as the filament and biofilm formation by *C. albicans*. Also, using qRT-PCR studies, we explored whether indole directly influences fungal virulence via *NRG1*, the transcriptional factor that influences filamentation and biofilm formation in *C. albicans*. Finally, we used the established model host *Caenorhabditis elegans* (reviewed in (Irazoqui *et al.* 2010)) to study the role of these molecules in eukaryotic-prokaryotic competition *in vivo*.

Materials and methods

Strains

The *C. albicans* strains used in this study were DAY185 (*ura3Δ::λimm434/ura3Δ::λimm434 ARG4:URA3::arg4::hisG/arg4::hisG his1::hisG::pHIS/his1::hisG*) as the wild-type strain (Davis *et al.* 2000) and the *efg1Δ/cph1Δ* double mutant (*ura3Δ::λimm434/ura3Δ::λimm434 cph1Δ::hisG/cph1Δ::hisG efg1Δ::hisG/efg1Δ::hisG-URA3-hisG*) (Lo *et al.* 1997). Yeast strains were grown in yeast-peptone-dextrose (YPD) or on brain heart infusion (BHI; Difco, Detroit, MI, USA) agar containing kanamycin (50 μg ml^{-1}) at 30°C (for the *C. elegans* solid killing assay). The *E. coli* OP50 (Breger *et al.* 2007) was routinely cultured in Luria-Bertani (LB) medium (Difco) at 37°C.

In the nematode assays, we used the *C. elegans* wild-type strain N2 Bristol, the strain CF512 *fer-15(b26);fem-1 (hc17)*, for *C. elegans* killing assay, and the strain AU37 *glp-4(bn2);sek-1(km4)* for *C. elegans* filamentation assay. These strains have been described in previous papers (Murphy *et al.* 2003; Kim and Mylonakis 2011) and nematodes were cultured and maintained on *E. coli* OP50 using standard procedures (Brenner 1974; Breger *et al.* 2007).

Silicone pad biofilm assay

The effect of indole and IAN on *C. albicans* biofilm growth was evaluated using a silicone pad assay, as described previously (Kim and Mylonakis 2011). In brief, the biofilm mass was calculated by subtracting the original weight of the silicone pad from its postincubation weight at 60 h and adjusted for the weight of control silicone pads not exposed to fungal cells. Spider medium was used in the *C. albicans* biofilm assays.

Candida albicans attachment assay

The human intestinal epithelial HT-29 cell line was obtained from the Korea Cell Line Bank (KCLB, Seoul, Korea). Cells were routinely cultured in RPMI 1640 medium (Gibco BRL, Rockville, MD, USA) supplemented with 10% heat-inactivated foetal bovine serum (FBS). *Candida albicans* attachment experiments were performed as described previously (Kim *et al.* 2009) with some modifications. Prior to the attachment assay, the HT-29 monolayers were washed three times with PBS to remove culture medium and nonattached cells. The prepared *C. albicans* strains (3×10^2 cells ml^{-1}), with 1 mmol l^{-1} indole or IAN, were added to the monolayers at 37°C in an atmosphere of 5% CO_2. After allowing 6 h, the monolayers were washed six times with PBS to remove non-attached bacteria, and the attached cells were collected using a cell scraper. Serial dilutions of the mixture were plated onto YPD agar containing kanamycin and incubated at 37°C for 48 h.

Filamentation assay using *Caenorhabditis elegans*

The *C. elegans* coinfection assays for *C. albicans* filamentation were performed as described in (Kim and

Mylonakis 2011). Briefly, young adult nematodes ($n = 20$) were infected for 4 h on lawns of *C. albicans* DAY185 and then transferred into wells (six-well microplate). Each well contained 2 ml of liquid assay medium (20% BHI and 80% M9) containing 0·2 mmol l^{-1} indole or IAN. The plates were then incubated at 25°C and examined for 5 days for the formation of penetrative filaments.

Caenorhabditis elegans solid killing assay

The *C. elegans* assays were performed using the protocols described by (Pukkila-Worley *et al.* 2011). In brief, 10 μl of an overnight *C. albicans* DAY185 culture was spread onto a BHI agar (that containing 0·2 mmol l^{-1} indole or IAN as needed). Then, young adult *fer-15;fem-1* worms ($n = 30$) were infected by placing them on the lawns (three assay plates were used per condition). Animals were incubated at 25°C and scored as live or dead on a daily basis by gently touching them with a platinum wire. Worms that crawled onto the walls of the tissue culture plates were eliminated from the analysis.

Measuring fungal colonization in the intestinal tract of *Caenorhabditis elegans*

The numbers of fungal cells in the worms intestine were determined as previously described (Garsin *et al.* 2001) with minor modifications. More specifically, at day 5, each worm was removed to a new sterile tube containing M9 medium with 1% Triton X-100 and was mechanically disrupted using a pestle (Kontes, Vineland, NJ, USA). The concentration of yeast cells was evaluated by diluting cells from 10^0 to 10^7 via 10-fold serial dilution steps in 0·85% NaCl solution; these dilutions were then plated on YPD agar containing kanamycin (50 μg ml^{-1}), ampicillin (100 μg ml^{-1}) and streptomycin (100 μg ml^{-1}) (Kim *et al.* 2010). Plates were incubated at 30°C for 48 h.

Quantitative real-time PCR (qRT-PCR)

The qRT-PCR assays were performed using the Applied Biosystems 7300 Real-Time PCR system (Applied Biosystems, Foster, CA, USA). Following mechanical disruption with zirconia/silica beads (Biospec, Bartlesville, OK, USA) and a pestle in Trizol solution (Invitrogen, Carlsbad, CA), total RNA samples from *C. albicans* were purified using a RNeasy Mini kit according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). After isolating RNA, 50 ng of total RNA was used for the qRT-PCR reaction using the *Power* SYBR® Green RNA-to-C_T™ *1-Step* kit (Applied Biosystems). The primers sequences used were as follows: *HWP1* (5′-CTCCAGCTGGCTCAAGTGGT-3′ and 5′-TGGCAGATGGTTGCATGAGT-3′), *NRG1* (5′-CACCTCACTTGCAACCCC-3′ and 5′-GCCCTGGAGAT GGTCTGA-3′), *LIP2* (5′-GGCCTGGATTGATGCAAGAT-3′ and 5′-GGCCTGGATTGATGCAAGAT-3′), *ALS3* (5′-ACTTCCACAGCTGCTTCCAC-3′ and 5′-TGCAGATGG AGCATTACCAC-3′) and 18s rRNA (5′-GTGCCAGCAG-CCGCGGTA-3′ and 5′-TGGACCGGCCAGCCAAGC-3′). Relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). Expression levels of the control gene 18S rRNA were used to normalize the expression data for *C. albicans*.

Statistical analysis

Candida elegans survival is presented using the Kaplan–Meier method and the significance of differences in survival was determined using the log-rank test (STATA6; STATA, College Station, TX, USA). Differences between experiments were determined using Student's *t*-test. Each result shown is representative of at least two independent experiments. A *P*-value of 0·05 in all replicates experiments was considered statistically significant.

Results

Indole and IAN dramatically inhibit biofilm formation by *Candida albicans*

Initially, to investigate whether indole signalling molecules influence the viability of *C. albicans* DAY185, growth curves were measured in the presence or absence of indole and IAN in YPD. As expected, there was no significant difference in the growth curves of *C. albicans* exposed to 1 mmol l^{-1} indole or IAN compared to the *C. albicans* alone (data not shown); hence, we indicated indole and IAN do not affect the viability of *C. albicans*. And then, we evaluated whether indole and IAN influenced *C. albicans* biofilm formation using the standard silicone pad assay. As shown in Fig. 1, C. *albicans* biofilm formation was dramatically repressed (by approximately 80% compared to control) by the presence of indole and IAN. These observations confirm that the bacterial signalling molecules indole and IAN influence fungal biofilm formation and/or fungal attachment.

Indole and IAN reduce the ability of *Candida albicans* attachment

In this series of experiments, we explored whether the bacterial signalling molecules indole and IAN affect the attachment of the fungal pathogen *C. albicans* to HT-29 intestinal epithelial cells. The presence of indole and IAN did not alter the viability of the HT-29 cell monolayer

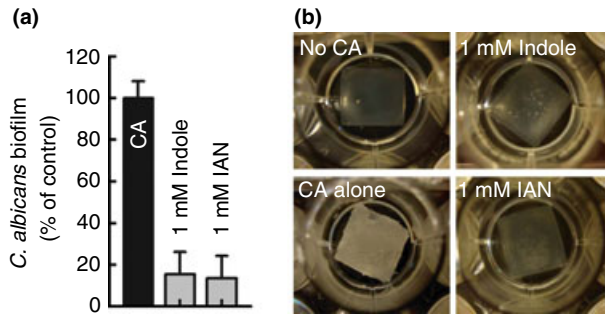

Figure 1 Indole and indole-3-acetonitrile (IAN) repress fungal *Candida albicans* biofilm formation. (a) Normalized *C. albicans* DAY185 biofilm assays were performed on silicone squares in spider medium for 60 h at 37°C. (b) Visualization of *C. albicans* DAY185 biofilm under the same conditions (No CA, no *C. albicans* treatment; CA alone, *C. albicans* alone; 1 mmol l^{-1} indole, *C. albicans* exposed to 1 mmol l^{-1} indole; 1 mmol l^{-1} IAN, *C. albicans* exposed to 1 mmol l^{-1} IAN).

over a period of 6 h (data not shown), but indole and IAN significantly reduced the attachment of *C. albicans* to HT-29 cells (Fig. 2a).

Indole and IAN inhibit the filamentation and attenuate virulence of *Candida albicans* in *Caenorhabditis elegans in vivo* by decreasing the fungal burden in the nematode intestine

Moreover, we evaluated *C. albicans* filamentation in the presence of indole and IAN *in vivo* using the established model *C. elegans*. Corroborating the biofilm and attachment results, filamentation in the presence of the indole and IAN was significantly less than that of the *C. albicans* control ($P = 0{\cdot}025$ and $P = 0{\cdot}010$ for indole and IAN, respectively, compared to worms exposed to *C. albicans* DAY185 alone) (Fig. 2b).

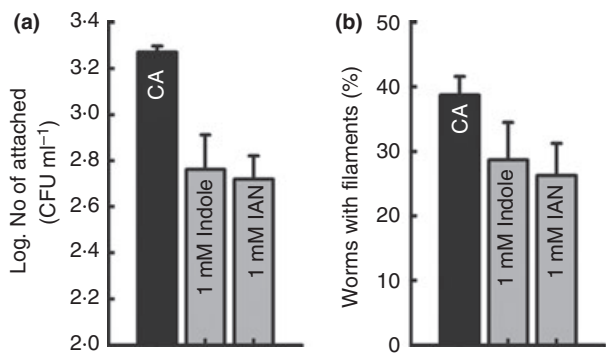

Figure 2 Indole and indole-3-acetonitrile (IAN) inhibit fungal *Candida albicans* attachment and filamentation. (a) Attachment of *C. albicans* DAY185 in the presence of 1 mmol l^{-1} indole or IAN to the HT-29 cell surface. *Candida albicans* DAY185 in antibiotic-free medium were exposed for 6 h at 37°C in a 5% CO_2 atmosphere. After incubation, the HT-29 cells were washed six times in PBS and plated on YPD agar containing kanamycin. (b) Inhibition of *C. albicans* DAY185 filamentation exposed to 0·2 mmol l^{-1} indole or IAN in the *Caenorhabditis elegans* coinfection model. Error bars indicate the standard deviation (SD) of independent experiments.

Using a solid killing assay, we showed that infection of *C. elegans fer-15(b26);fem-1(hc17)* with *C. albicans* DAY185 was lethal within 7 days, whereas nematodes infected with the attenuated *efg1Δ/cph1Δ* double mutant strain of *C. albicans* were not killed. Interestingly, the presence of indole and IAN significantly enhanced the resistance of *C. elegans* to infection with *C. albicans* (Fig. 3a; $P = 0{\cdot}0188$ and $P < 0{\cdot}0001$ for indole and IAN, respectively, compared to worms exposed to *C. albicans* DAY185 alone). Similar results were obtained with N2 wild-type worms (data not shown). Because *C. albicans* colonizes the nematode intestinal tract and causes a persistent lethal infection (Breger *et al.* 2007), we evaluated the possibility that indole and IAN decreased the number of persistent *C. albicans* in the *C. elegans* intestine by assessing the number of colony forming units (CFU) of *C. albicans* in the worm intestines. We found that worms treated with indole or IAN had significantly fewer *C. albicans* cells present in their intestines after 5 days compared to control worms (Fig. 3b).

Indole and IAN specifically stimulate the transcription of *NRG1*

Based on the findings above that indole and IAN are involved in the regulation of several biofilm-associated factors, we examined the expression levels of *C. albicans* genes involved in adherence as well as filament and biofilm formation. As shown on Fig. 4, qRT-PCR assays showed that *NRG1* expression was upregulated by indole and IAN ($4{\cdot}3 \pm 1{\cdot}7$-fold for indole and $7{\cdot}6 \pm 1{\cdot}8$ for IAN, respectively), whereas there was no significant difference in the expression levels of *HWP1*, *LIP2* and *ALS3*. Therefore, our qRT-PCR results suggest that, at least in part, indole and its derivatives may inhibit fungal virulence by altering the expression of *NRG1*.

Discussion

Bacterial indole signalling is important for controlling microbial biofilm formation by pathogenic *E. coli* (Lee *et al.* 2011) and *Vibrio cholerae* (Mueller *et al.* 2009). Although we previously showed that bacterial signalling molecules can influence the attachment ability of pathogenic bacteria (Kim *et al.* 2007), a few study has investigated the role of these signalling molecules in the adherence of yeast cells. In the current study, we confirmed that indole is an interactive regulator between

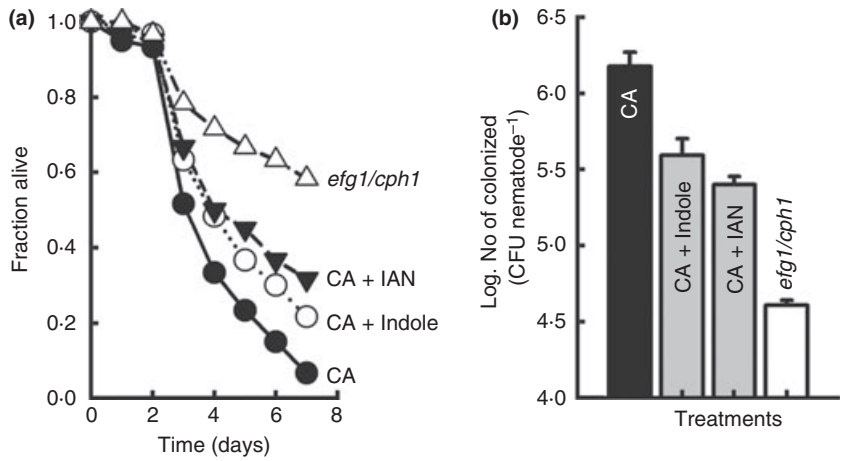

Figure 3 Indole and indole-3-acetonitrile (IAN) prolong the survival of *Caenorhabditis elegans* infected with *Candida albicans* by decreasing fungal burden in the nematode intestine. (a) Solid killing assays (n = 30 per plate) of *C. elegans* strain *fer-15;fem-1* infected with *C. albicans* DAY185 in the presence of 0·2 mmol l^{-1} indole or IAN (Survival statics: P = 0·0188 for indole, P = 0·0013 for IAN and P < 0·0001 for the *efg1Δ/cph1Δ* mutant, respectively, compared to worms exposed to *C. albicans* DAY185 alone). (b) Fungal colonization (CFU per nematode) of *C. albicans* DAY185 exposed to 0·2 mmol l^{-1} indole or IAN in the *fer-15;fem-1* nematode intestine. Two independent biological replicates were performed for each experiment. Error bars represent standard deviations.

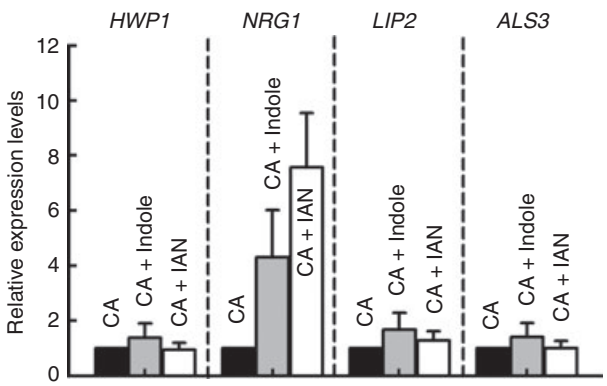

Figure 4 Indole and indole-3-acetonitrile (IAN) stimulate the transcription of *NRG1*. We performed qRT-PCR analysis to evaluate the impact of indole and IAN on the transcription of genes associated with biofilm formation by *Candida albicans*. Transcript levels were measured in *C. albicans* DAY185 in the presence of 1 mmol l^{-1} indole or IAN for 6 h. The housekeeping gene 18S rRNA was used to normalize gene expression.

bacteria and fungi, and showed that the anti-fungal activity of indole and its derivative IAN is related to the regulation of *NRG1*. In this work, we provide multiple lines of evidence that indole and IAN directly regulate fungal virulence: (i) indole and IAN did not influence fungal growth, (ii) indole signalling decreased fungal virulence *in vitro* as shown in biofilm, attachment and filamentation assays (Figs 1 and 2) and *in vivo* as shown in the *C. elegans* killing assay (Fig. 3) and (iii) *NRG1* expression was altered by indole signalling (Fig. 4). Hence, indole plays a critical role in specific bacteria–fungi interaction networks as a cross-kingdom signalling molecule.

Medically and environmentally important interactions between bacteria and fungi are common in nature (Peleg *et al.* 2010). Cell-to-cell communication, which is mediated by signalling molecules, is critical in polymicrobial communities. Although the intra- and inter-species-specific behaviours of these molecules have been investigated (Miller and Bassler 2001), only a few studies have examined cross-kingdom signalling. Reen and colleagues (Reen *et al.* 2011) showed that PQS and HHQ, which are 4-quinolone signalling molecules produced by *Pseudomonas aeruginosa*, significantly repressed the biofilm formation by fungi (*C. albicans*) as well as bacteria (*Bacillus subtilis*). These molecules are not naturally present in the human intestine and are toxic. However, a variety of both Gram-positive and Gram-negative bacteria (more than 80 species) can produce indole, and indole signalling is involved in spore formation, drug resistance, virulence, plasmid stability and biofilm formation in multi-species bacterial communities (Lee and Lee 2010). We showed that indole and IAN directly modulate the virulence of *C. albicans*. It is reasonable to assume that a high concentration of these signalling molecules is physiologically present in the host intestine. For example, strains of *E. coli* can produce up to 0·6 mmol l^{-1} indole, which is not toxic to the host (Chant and Summers 2007).

Consistent that filament formation is critical for *C. albicans* virulence (Lo *et al.* 1997) involved in biofilm formation (Nobile and Mitchell 2005; Richard *et al.*

2005), we showed indole and IAN repressed *C. albicans* filamentation using the established *in vivo* model *C. elegans* (Breger *et al.* 2007; Pukkila-Worley *et al.* 2009; Tampakakis *et al.* 2009; Kim and Mylonakis 2011). Here, we employed the low, nontoxic concentration of 0·2 mmol l^{-1} indole or IAN for the *C. elegans* killing assay as indicated previously (Anyanful *et al.* 2005) as 0·5 or 1 mmol l^{-1} concentrations of indole or IAN were toxic to *C. elegans* (data not shown). Equally interesting, under same conditions, we showed indole or IAN significantly attenuates the toxicity of *C. albicans*. Taken together, these studies demonstrate that bacterial indole and IAN inhibit fungal attachment and filamentation as well as biofilm formation, and prolong the survival of nematodes exposed to fungal *C. albicans* by decreasing the burden of *C. albicans* infection in the intestine, and decreasing *C. albicans* virulence.

Based on the findings presented in this report, indole can be considered to be an inter-kingdom signalling molecule as well as an inter-genus and inter-species signalling molecule. Previously, it has been well established that several genes included *HWP1* (encodes a filament-specific cell wall protein) (Nobile *et al.* 2006), *NRG1* (encodes a transcriptional repressor and regulates filament formation and virulence) (Uppuluri *et al.* 2010), *LIP2* (encodes a biofilm-associated lipase) (Xie *et al.* 2011) and *ALS3* (encodes filament-specific adhesion) (Zhao *et al.* 2006) are important role in filament and biofilm formation of *C. albicans*. Unexpectedly, these factors were not involved in response to indole or IAN treatment. Interestingly, this effect is by affecting the transcription of *NRG1*, a gene that encodes a DNA-binding protein. Much has been learned that NRG1 critically appears to influence all these aspects of *C. albicans* pathogenesis (Braun *et al.* 2001; Murad *et al.* 2001). As recently shown (Uppuluri *et al.* 2010), *NGR1* gene expression is involved in *C. albicans* biofilm formation and dispersal. Based on previous results, we speculated that indole cross-kingdom signalling would affect *NRG1* transcription; we found that *NRG1* transcription was induced by indole and IAN, resulting in a significant reduction in fungal filamentation and biofilm formation. As noted above, in *C. albicans*, biofilm formation is playing critical roles in their virulence (Richard *et al.* 2005) and is involved in two major processes, (i) attachment to biotic or abiotic surface and (ii) morphological transition from yeast to filaments are major requirements for biofilm formation (Blankenship and Mitchell 2006).

To our knowledge, this is first report that bacterially produced indole controls fungal virulence by regulating the transcription of NRG1 gene, a transcriptional factor. Our findings support the idea that indole-related signalling molecules may play an important role in inter-kingdom regulatory networks that are relevant to human health.

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