Bacterial Derived Carbohydrates Bind Cyr1 and Trigger Hyphal Growth in *Candida albicans*

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Supporting Information

**ABSTRACT:** The dimorphic yeast *Candida albicans* is the most common pathogenic fungus found in humans. While this species is normally commensal, a morphological switch from budding yeast to filamentous hyphae allows the fungi to invade epithelial cells and cause infections. The phenotypic change is controlled by the adenylyl cyclase, Cyr1. Interestingly, this protein contains a leucine-rich repeat (LRR) domain, which is commonly found in innate immune receptors from plants and animals. A functional and pure LRR domain was obtained in high yields from *E. coli* expression. Utilizing a surface plasmon resonance assay, the LRR was found to bind diverse bacterial derived carbohydrates with high affinity. This domain is capable of binding fragments of peptidoglycan, a carbohydrate polymer component of the bacterial cell wall, as well as anthracyclines produced by *Streptomyces*, leading to hyphal formation. These findings add another dimension to the human microbiome, taking into account yeast–bacteria interactions that occur in the host.

**KEYWORDS:** *C. albicans*, anthracycline, peptidoglycan, hyphae formation, leucine-rich-repeat domain

The human body plays host to trillions of microorganisms collectively referred to as the microbiome. It has been reported that there are as many bacteria as human cells in the average individual. While most literature examining the microbiome focuses on these bacterial species, there is a smaller yet no less important population of fungal species, the “mycobiome”, present in all individuals. Similar to host immune cells, these fungi can recognize changes in the microbiome composition often leading to disease states. *Candida albicans* is perhaps the most recognizable fungal infection, as either oral candidiasis (or “thrush”) or vaginal candidiasis commonly referred to as a “yeast infection”, which affects nearly 75% of women at least once in their lifetime. *Candida* infections can also enter the bloodstream, where patient outcomes are much worse. *Candida* species are responsible for 9% of hospital acquired bloodstream infections with mortality rates at approximately 20%. To effectively treat these infections, it is important to understand how these fungi are turning pathogenic.

*C. albicans* is a dimorphic fungus capable of growing as budding yeast or as filamentous hyphae. It is the hyphae form which allows *C. albicans* to form biofilms and become invasive, by either endocytosis by the host cell or active penetration of the plasma membrane by the hyphae. Several pathways including the cAMP-PKA, MAPK, cell cycle arrest, and pH pathways regulate the morphogenesis of *C. albicans*. The cAMP-PKA pathway is of central importance, as Rocha et al. have demonstrated that knocking out the *CaCDC35* gene (also known as *CaCYP1*), which encodes the adenyl cyclase, Cyr1, prevents hyphal formation in *C. albicans*. Cyr1 responds to a number of signals including G-protein receptors, RAS proteins, elevated temperatures, CO2 levels, quorum sensing molecules, farnesol, homoserine lactone, and serum. Although serum has been known since the 1950s to elicit hyphae formation, it was not until 2008 that the compound responsible for hyphal growth was identified as fragments of peptidoglycan, the carbohydrate polymer which forms the bacterial cell wall. This proved especially interesting, as studies have indicated that *C. albicans* infections increase with fluctuations in microbiome composition, especially those caused by antibiotic treatment.

Cyr1 contains a leucine-rich repeat (LRR) domain, an evolutionary conserved motif common in many innate immune receptors found in both mammals and plants. Like many of these receptors, Cyr1 is able to bind to peptidoglycan fragments, specifically muramyl dipeptide (MDP) (Figure 1). Xu et al. demonstrated this interaction using a streptavidin pull down assay employing biotinylated MDP. This work provided a molecular identity to the hyphae inducing agent in serum.

Peptidoglycan is a polymer of the carbohydrates, N-acetylmuramic acid and N-acetylglucosamine. The latter of these is used exclusively in bacterial peptidoglycan, thus making it unique to bacteria. Interestingly, fragments of the bacterial cell
wall are not the only carbohydrate containing compounds known to affect the morphology of *C. albicans*. In 2010, Kwok et al. showed that DNA topoisomerase inhibitors, specifically anthracyclines, altered the morphology of *C. albicans*. The ability of anthracyclines, such as doxorubicin (2, Figure 1), to induce *C. albicans* hyphae formation is intriguing as the compounds are natural products produced by *Streptomyces peucetius* and contain the L-sugar, daunosamine (3, Figure 1). Chemotherapy has been linked to an increased risk for systemic Candida infections in cancer patients. We hypothesized that the bacterial derived carbohydrate could play a role in this phenotype and sought to establish a robust biochemical assay to probe the importance of the carbohydrate.

\[ \text{Percent Hyphae} \]

| Condition     | Hyphae Percentage |
|---------------|-------------------|
| Non Induced   | 6.14%             |
| 10% FBS       | 36.77%            |
| MDP           | 29.13%            |
| MTP           | 41.43%            |
| Daunosamine   | 6.83%             |
| Doxorubicin   | 7.49%             |

**Figure 1.** Bacterial derived carbohydrates. A diverse set of carbohydrates were chosen to test for their ability to induce hyphae formation in *C. albicans*. MDP (1) and MTP (4) are fragments of the bacterial peptidoglycan. Daunosamine (3) is the sugar moiety of the *Streptomyces peucetius* natural product and chemotherapeutic agent doxorubicin (2).

![Figure 1](image1.png)

**Figure 2.** Bacterial derived carbohydrates elicit hyphae formation in *C. albicans*. 1 mL *C. albicans* cultures were grown overnight at 37 °C in YPD media in the presence of 10% fetal bovine serum (FBS) or 1 mM of the indicated compound. Cells were scored for hyphal growth and reported as a percentage. Specific details regarding scoring cells can be found in the Methods.

**Scheme 1. Synthesis of Muramyl Tripeptide (Lysine)**

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Reagents and conditions: (a) 1. HBTU, N-methylmorpholine, DMF, 2. protected tripeptide, 64%; (b) Pd/C, H2O, MeOH, AcOH, 95%.
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In this work, we expanded the scope of bacterial derived carbohydrates capable of eliciting a hyphae response in *C. albicans*. Further, we report an improved method for expressing and purifying the LRR of Cyr1 in *Escherichia coli*, resulting in high yields and removing contaminating peptidoglycan fragments. Finally, using a surface plasma resonance (SPR) assay, previously developed in our lab to characterize the NOD2–MDP interaction, we characterize the ability of the LRR domain to bind a subset of bacterial derived carbohydrates.

**RESULTS AND DISCUSSION**

A binding assay for a LRR containing protein, Nod2, and a peptidoglycan fragment was established in our laboratory.
this assay, the peptidoglycan fragment was linked via an amine handle, which was installed on the carbohydrate backbone. However, in the initial binding experiments between MDP and Cyr1, a biotin affinity handle was added to the peptide portion of the peptidoglycan fragments. These data suggest that a quantitative binding assay could support modifications on the peptide. Here, a synthesis of a new peptidoglycan fragment, muramyl tripeptide (lysine) (MDP) (4, Figure 1), was developed. Gram-positive bacteria naturally incorporate a lysine at the third position of the pentapeptide in their peptidoglycans. It was reasoned that the amine on the lysine would serve as an attachment point for the SPR assay (Figure 2A). Boons and co-workers established a novel chemistry for producing such fragments on a solid phase. Recently, we have developed robust solution chemistry to access amino versions of peptidoglycan fragments which utilizes a benzyl protected carbohydrate (5, Scheme 1). Here, a tripeptide (7, Scheme 1), which contains benzyl protecting groups, was synthesized using solution based chemistry (see the Supporting Information for synthetic details; Supplemental Scheme 1). 7 was coupled to 5 using coupling reagents to minimize epimerization, and the resulting product was globally deprotected using catalytic hydrogenation to yield 4, a novel MTP whose tripeptide contains l-alanine, d-isoglutamine, and l-lysine. As in many peptidoglycans found in nature, the isoglutamine in 4 is linked to the lysine via the side chain as opposed to the peptide backbone and the α-carboxyl group of glutamic acid is amidated. Boons and co-workers have shown that MTPs retained the ability to activate an immune response in humans; however, it was not known if MTPs would activate the hyphal response in yeast.

Compound 4 was tested for the ability to induce hyphal growth in C. albicans (Figure 2) along with a known activator, MDP (1, Figure 1). 1 induced hyphal formation, in agreement with the previously published results. However, 4 proved to be more potent in activating hyphal growth, while also appearing to initiate biofilm formation (Supplemental Figure 1). These data show that MTPs are capable of activating a response in yeast.

In addition to probing peptidoglycan carbohydrates, the activity of doxorubicin (2, Figure 1), a glycosylated anthracycline produced by bacteria, and its associated carbohydrate, daunosamine (3, Figure 1), to activate hyphal growth was screened. 2 triggered a slight increase in hyphal growth but also caused cell death, which can be expected as 2 is a topoisomerase inhibitor. Interestingly, 3, which is only the carbohydrate component, was also able to mildly induce hyphal formation without the associated cell death seen with the parent compound, 2. These results suggest that the carbohydrates from peptidoglycan and bacterial derived small molecules are important for inducing hyphal growth.

Intrigued by the results of the hyphal growth assay, the ability for the carbohydrates to bind to Cyr1 was assessed. To accomplish this, a robust purification of the LRR domain of Cyr1 had to be developed. Previous work from Xu et al. established purification of a GST-LRR fusion protein, which was used to demonstrate LRR-MDP binding via a biotin-streptavidin pull down assay and subsequently resolved via Western blot analysis. However, in our hands, this method did not give high enough yields of protein to use in the desired quantitative binding assay. A hexahistidine-tagged maltose-binding protein (MBP) fusion protein, which is known to increase protein solubility, was added to the N-terminus. Despite the addition of the MBP tag, the protein remained largely insoluble when expressed in E. coli. Alterations in expression and lysis conditions were not successful in increasing yields of the soluble fusion protein. Cyr1-LRR could be precipitating as it is expressed in the presence of a known ligand, peptidoglycan fragments. Therefore, a denaturation-refolding technique was employed to extract the fusion protein from inclusion bodies produced during protein expression. These conditions would ensure that any bound peptidoglycan fragment/ligand would be released. Using this method, MBP fused to Cyr1’s LRR was obtained in good yield (10 mg/L of expression) with ~90% purity (Supplemental Figure 2A). The protein showed a narrow range of stability as it aggregated when dialyzed into a buffer below pH 6.0 (Supplemental Figure 2B), which was important for developing the SPR binding assay. The refolding of the protein was confirmed by circular dichroism (CD). Minima at 208 and 222 nm indicated that the fusion protein contained high α-helical character, consistent with the structure of the LRR domain of homologous proteins. To ensure that secondary structure was conserved during the refolding process, a CD spectrum of MBP-LRR that was expressed in the soluble fraction was obtained and compared to the spectrum of the refolded protein (Supplemental Figure 3). Both spectra show a similar minimum, indicating the protein was able to refold with the proper secondary structure. Protein identity was further confirmed by LC-MS/MS (Supplemental Figure 4). Using a MBP tag and a
denature/renature purification procedure, Cyr1’s LRR was obtained in large quantity and high purity.

With the purified protein in hand, an assay was developed to determine if the compounds capable of inducing hyphae growth (Figure 2) could bind to the LRR domain of Cyr1. Our laboratory has developed a surface plasmon resonance (SPR) assay,2426 which has been used to measure the binding of bacterial peptidoglycan fragments to the LRR domain of the innate immune receptor NOD2.25 This assay was modified to determine the affinity of the LRR from Cyr1 to the bacterial derived carbohydrates. Briefly, the SPR analysis employed involves covalently attaching a ligand of interest to a self-assembled monolayer (SAM) attached to a gold chip (Figure 3A). The constructed SAM contained MTP (4), daunosamine (3), and ethanolamine; this chip was utilized to probe the LRR’s ability to bind the various carbohydrates. The purified MBP-LRR was applied to the ligand-modified surface, and changes in refraction index were monitored in order to generate a SPR sensogram (Supplemental Figure 5). Using this SPR assay, it was determined that the LRR bound to 4 with a $K_D$ of $176 \pm 68$ nM and to 3 with a $K_D$ of $287 \pm 88$ nM (Figure 3B). These data demonstrate that the LRR is capable of binding diverse carbohydrates with high affinity. To verify that the LRR is responsible for the binding event, the free MBP tag was purified as a control and applied to the chip. The free MBP tag was unable to bind either compound (Supplemental Figure 6) suggesting that the observed binding is specific to the LRR.

To validate the observed $K_D$ was due to Cyr1-LRR affinity for the carbohydrates and not the result of nonspecific absorption to the chip, a competition assay was performed. Cyr1-LRR was preincubated with increasing concentrations of 3 or 4, ranging from the approximate $K_D$ up to 64-fold the $K_D$. The protein was applied to the SPR chip, and the response was recorded and compared to the response levels of the apo Cyr1-LRR. Preincubation with 4 reduced the binding response to 3 and 4 by 49.5% and 54.5%, respectively (Figure 4A). Preincubation with 3 was as effective with response to 3 and 4 at 56.3% and 50.5% of apo Cyr1-LRR binding levels (Figure 4B). With confirmation that the SPR signals were the result of Cyr1-LRR specifically binding to the carbohydrates, the competition experiment was repeated with 1 and 2 to probe their ability to bind Cyr1-LRR and inhibit binding to the immobilized carbohydrates. MDP (1, Figure 1) was capable of reducing binding of Cyr1-LRR to 3 and 4 by 55.4% and 58.5% of apo levels, respectively (Figure 4C). Preincubation with doxorubicin (2, Figure 1) reduced responses to 3 and 4 by 61.4% and 66.9%, respectively (Figure 4D). These data demonstrate the Cyr1’s LRR has a versatile binding site. Cyr1 contains 14 LRRs, and the data indicate a single binding site within this domain, as 1–4 are capable of reducing binding to peptidoglycan or anthracyclic associated carbohydrates. To ensure the decrease in binding affinity was not a result of nonspecific interactions, CYR1-LRR was incubated with alanine, glutamine, or chloramphenicol. These compounds did not decrease the response compared to the apo Cyr1-LRR (Supplemental Figure 7). However, in order to fully appreciate Cyr1-LRR’s carbohydrate binding pocket, future experiments, either mutational in nature or synthetic (i.e., photoactivatable cross-linkers), are needed to determine the molecular makeup of Cyr1’s carbohydrate binding residues.

The development of a robust E. coli based expression and refolding purification protocol grants access to sufficient quantities of the LRR domain of Cyr1, free from contaminating natural ligands that are present when the protein is expressed in a bacterial host. Using a quantitative binding assay, Cyr1’s LRR domain was found to bind a diverse range of carbohydrates from bacterial sources with high affinity, highlighting the importance of the interactions between yeast and bacteria in the human microbiome. The development of this assay will allow for the potential identification of compounds that inhibit carbohydrate binding and subsequent hyphae formation, ultimately, leading to novel therapeutics, which can be coadministered with antibiotics or chemotherapeutics to prevent systemic C. albicans infections.
METHODS

For full experimental details regarding materials, synthetic protocols, protein expression and purification, SPR procedures, and the hyphal activation assay, refer to the Supporting Information.

Synthesis of MTP. Standard peptide coupling conditions were used to couple the tripeptide to the protected muramic acid. Global deprotection was performed using Pd/C and H2. MTPs were purified using a Waters auto purification system. For detailed synthetic protocols, including 1H and 13C NMR and high-resolution mass spectra, please see the Supporting Information.

Hyphal Growth Assay. Ten mL of yeast peptone dextrose (YPD) was inoculated with C. albicans (SC5314) from a glycerol stock stored at −80 °C. The culture was grown overnight at 30 °C. The cells were separated into 0.9 mL aliquots, and 100 µL of sterile H2O or 10 mM MTP (4), MDP (1), daunomycin (3), or doxorubicin (2) was added. Cultures were incubated overnight at 37 °C while shaking at 200 rpm. Aliquots were taken and imaged at 40x magnification. Images containing approximately 100 cells were blindly scored for hyphal growth. Hyphal growth was determined by dividing cells displaying hyphal characteristics by total cells. Percentages reported are the average of two independent trials.

Expression and Purification of Constructs. The MBP-LRR was expressed at 30 °C until an optical density (600 nm) of 0.6–0.8 was reached. Protein expression was induced by the addition of IPTG to a concentration of 1.0 mM overnight at 18 °C. A pellet from 1 L of cells was resuspended and lysed using sonication. The inclusion body pellet was washed and denatured. A clarified inclusion body suspension was applied to the 5 mL HisTrap HP column (GE LifeSciences) on a NGC Quest 10 chromatography system (Bio-Rad). After washing with low amounts of imidazole, the protein was eluted with high concentrations of imidazole. Fractions containing the MBP-LRR were pooled and dialyzed overnight into refolding buffer with high concentrations of arginine. Refolded protein was dialyzed into Biacore assay buffer. Protein concentration was determined by absorbance at 280 nm (ε = 91 595 M⁻¹ cm⁻¹). For detailed protein purification protocols, including SDS-PAGE images, CD spectrum, and mass spectra, please see the Supporting Information.

Surface Plasmon Resonance Assay. Methods for preparing the chip, mixed-SAM, and SPR assay conditions were adapted from Grimes et al. The Biacore 3000 instrument was used for all SPR assays described. Full experimental details and raw sensograms are presented in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.7b00154.

Supplemental methods such as materials, expression and purification of LRR-CRY1, SPR conditions, hyphal growth assay, synthetic procedures, and NMR data (PDF)

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Author Contributions

The study was designed by J.M.B., D.D.W., and C.L.G.; J.M.B. and S.M. conducted the experiments. All authors analyzed the data. J.M.B. and C.L.G. wrote the manuscript, and all authors have read and given approval to the final version.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

MDP, muramyl dipeptide; LRR, leucine-rich repeat; SPR, surface plasmon resonance; CD, circular dichroism; SAM, self-assembled monolayer; MTP, muramyl tripeptide; YPD, yeast peptone dextrose; FBS, fetal bovine serum

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