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Effect of the copper-responsive factor Cuf1 on the capsule biosynthesis in *Cryptococcus neoformans*

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Abstract: [Objective] *Cryptococcus neoformans* is an opportunistic human fungal pathogen that primarily infects immunocompromised patients. The most distinctive feature of *C. neoformans* is that it contains a highly regulated major virulence factor, the polysaccharide capsule. We demonstrate in this paper that a copper-dependent transcription factor gene *CUF*1 has a negative role in capsule biosynthesis. [Methods] Colony morphology, Indian inking staining microscopy, volume of cell suspension observation, weight of capsule produced by the disruption mutant of *CUF*1 and the wild type control. [Results] The mutant produced a mucoid large colonies compared to that of the wild type. Capsule size was larger as shown by microscopy. The mutant had a larger volume with the same number of cells. And the mutant produced more capsule as determined by weight of dried extracellular polysaccharides. Iron repletion could suppress the capsule overproduction phenotype of the Δ*cuf*1 mutant. [Conclusion] The copper responsive transcription factor 1 (Cuf1) negatively regulates capsule biosynthesis in *C. neoformans*. Cuf1 may act via modulating iron acquisition through the high-affinity iron uptake pathway.

Keywords: capsule; *Cryptococcus neoformans*; virulence factor

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Cryptococcus neoformans, an encapsulated basidiomycetous fungus, has become a common opportunistic human pathogen as a result of a rising immunocompromised population over the last two decades[1]. *C. neoformans* has several virulence factors that are essential for its survival *in vivo* [10], including a polysaccharide capsule, which is believed to provide protection against various environmental stresses and serve to aid in the evasion of the host immune response[9-12]. The importance of capsule production in cryptococcal pathogenesis is emphasized by the fact that capsule-deficient mutants have been shown to be avirulent[2-8].

Under *in vitro* growth conditions, the capsule size is usually small; while cryptococcal cells with markedly increased capsule size has routinely been observed in various infected host tissues[22-26, 29], suggesting that environmental conditions influence the cryptococcal capsule size. It is well established that capsule growth in *C. neoformans* can be dramatically induced in response to a variety of factors[20, 26-28], including low iron, high CO_2 concentrations, mammalian serum, during animal infection host etc[18-19, 33, 36]. In addition, a number of genes and their associated signaling transduction pathways, including the mitogen-activated protein kinase (MAPK) and cAMP signaling pathways, have been implicated in both capsule formation and virulence[2, 8, 14-16, 36]. Despite extensive research on the subject, it is still not clear how the capsule synthesis process in *C. neoformans* is directly regulated by the downstream effectors of the MAPK and cAMP signaling pathways. Complete description of the regulatory

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processes of capsule formation in molecular terms is one of the major goals of the cryptococcal research community. The aim of this paper was to determine the functional role of Cuf1 which encodes a copper-responsive transcription factor in *C. neoformans* capsule production. Here we provide conclusive evidence that Cuf1 plays a negative role in the biosynthesis of capsule. We further propose that Cuf1 may achieve its negative role in capsule production by effecting iron acquisition through modulating the high-affinity iron uptake pathway.

1 MATEREAL AND METHODS

1.1 Strains and Media

The *C. neoformans* serotype A strain H99 (ATCC 208821) was used as the wild type strain in this study. Both the Δ*cuf*1 strain (H99 *cuf*1::*URA*5), a knockout mutant of *CUF*1 and the complemented Δ*cuf*1 strain, which derived from reintroduction of the wild-type copy of *CUF*1 into the Δ*cuf*1 strain, were kindly provided by Dr. Peter Williamson. Yeast cells were normally grown on YPD agar (1% yeast extract, 2% peptone and 2% glucose, 2% agar) at 30℃. Maltose liquid or solid (supplemented with 2% agar) medium containing 0.2% soya peptone, 0.1% yeast extract and 2% maltose was used to induce capsule growth.

1.2 Determination of capsule size

The cells were cultured on maltose agar at 30℃ for at least 7 days. To visualize the size of the capsule, the cells were suspended in India ink solution (India ink: H_2O = 3:2), and examined under oil immersion (magnification, ×1000) light microscopy. Images of the cells were obtained using Eclipse 80i epifluorescent microscope system (Nikon, Tokyo, Japan) equipped with a 100× wide numerical aperture (1.30) Fluor objective lens and operated using Nikon NIS-Elements software. Images were captured by a Nikon DS-Ri1 digital cooled camera. At least three different fields were randomly chosen and photographed. The capsule size (distance from the edge of the capsule to the cell wall) and the cell body diameter of 30 cells from each strain were measured and averaged by NIS-Elements software. Cell fields shown in this study are representative of results repeated at least four times. In addition, data analyses were performed using the Excel spreadsheet software package.

1.3 Observation of the volume of cell suspension

A convenient and stable method to display capsule volume for a fixed number of cells was modified from D'souza *et al*.[37]. The cells were incubated as described above. 1.0×10^9 fresh cells were taken and washed twice with Na_3PO_4 (0.05 mol/L, pH 6.5), and resuspended in 1.0 ml Na_3PO_4 (0.05 mol/L, pH 6.5) or water in test tubes. The cell suspensions were allowed to stand straight for 3 hr. Tubes were then photographed.

1.4 Quantification of capsule polysaccharides

To determine quantitatively capsule polysaccharide produced by the same number of cells of each strain, capsular polysaccharide was extracted by following the description[24-30]. Cryptococcal cells were grown in liquid maltose media at 30℃ for 7 days and cells were pelleted by centrifugation at 10000 × g for 30 min. To 3 ml of the supernatant, 2 volume of ethanol and 1/10 volume 1 mol/L sodium acetate were added at 4℃ and incubated overnight. The polysaccharide was collected by centrifugation at the maximum speed with an Eppendorf bench top centrifuge. And the pellet was allowed to dry at room temperature, and dissolved in 3 ml of ddH_2O. Phenol extraction to remove proteins was carried out twice. The above steps were repeated once. The capsular polysaccharide was lyophilized and the weight of the capsule was determined by an analytical scale (Startorius, Germany). Quantification for each sample was carried out in triplicate.

2 RESULTS

2.1 Evidence of a negative effect Cuf1 on capsule production

To examine the effects of the deletion of *CUF*1 on capsule production in *C. neoformans*, wild-type H99 and Δ*cuf*1 were grown on maltose medium to induce the capsule formation. Colony morphology on maltose plates for 5 days was shown in Fig.1. The Δ*cuf*1 mutant produced mucoid colonies with apparently more capsule generated in comparison to H99 (Fig. 1-A). Due to more capsule produced, larger colonies were also observed for the mutant than that of the wild type although the incubation condition was the same. For further confirmation, microscopy was conducted to

observe the capsule formed by each strain by India ink stainning. As shown in Fig.1-B, the capsule formed by $\Delta cuf1$ cells was significantly bigger than that of H99 cells.

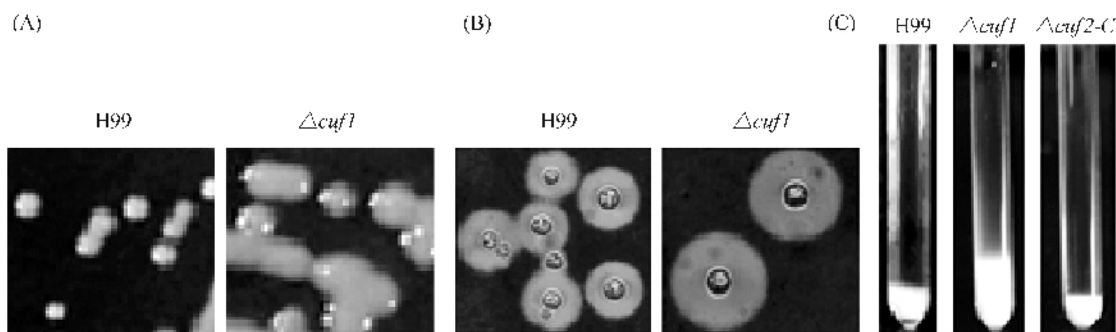

Fig.1 Increased capsule production by $\Delta cuf1$ mutant. A. Colony morphology of H99 and the mutant *cuf1* on maltose agar, indicating that more capsule was synthesized by the mutant. B. Increased capsule size was confirmed for $\Delta cuf1$ cells by microscopy (× 600). C. Volume of 1.0×10^9 cells in suspension. 1.0×10^9 fresh cells counted with haemocytometer and suspended 1 ml Na_3PO_4 (0.05 mol/L, pH 6.5) in test tubes. $\Delta cuf1$ has a larger volume than H99 and the complemented strain (*cuf1C*).

Capsule is a complex polysaccharide structure surrounding the *C. neoformans* yeast cells, with characteristic surface physicochemical properties. Thus, disparity in cell size due to capsule size differences will result in different interactions among cells and presumably will lead to different volume of cell suspension. We observed the volume of suspensions containing the same number of yeast cells from H99 and the mutant $\Delta cuf1$. As shown in Fig. 1-C, suspension of 1.0×10^9 cells of the $\Delta cuf1$ had a significantly larger volume in phosphate buffer while compared to the suspensions of H99 and the complement containing the same number of cells. The above results indicate that deletion of *CUF1* results in the increase of capsule biosynthesis.

2.2 Quantitative determination of capsule production in $\Delta cuf1$

The capsule size of 30 cells randomly picked by microscopy was measured using NIS-Elements software as described in Materials and Methods. For the wild-type H99 strain, the mean value of capsule size was 4.73 ± 0.24 μm, whereas the value was 7.18 ± 0.36 μm for the $\Delta cuf1$ cells which is about 1.52-fold larger (Fig.2-A). Importantly, the complemented *cuf1* C strain had a capsule size of 4.82 ± 0.33 μm, which is comparable to that of the H99 strain, indicating that the markedly increased capsule size in the $\Delta cuf1$ mutant was attributable to the deletion of *CUF1* .

The enlarged capsule phenotype in the $\Delta cuf1$ could be due to either increased capsular polysaccharide biosynthesis or a loose capsule structure or both. To gain better insights into the nature of the enlarged capsule size as a result of loss of Cuf1 function, capsule production in $\Delta cuf1$ and wild-type H99 was respectively quantified as described in Materials and Methods. As shown in Fig. 2-B, the mean quantity of dry capsule polysaccharide derived from 3 ml supernatant of $\Delta cuf1$ cultures was determined to be 182.86 ± 12.16 μg, which is approximately 1.5 times more than that of the H99 strain (122.86 ± 4.63 μg) or of the complemented *cuf1C* strain (118.55 ± 6.97 μg).

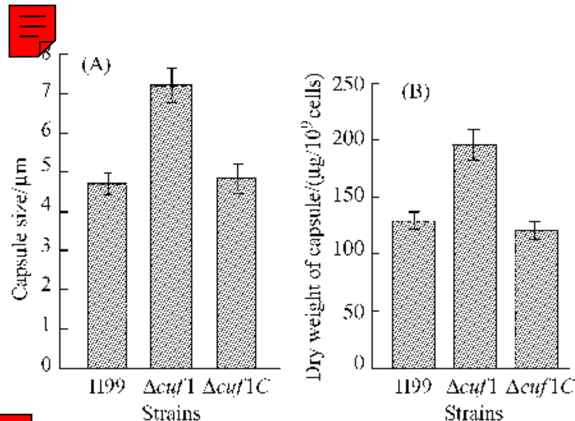

Fig.2 Quantitative analysis of capsule biosynthesis in $\Delta cuf1$ mutant. A. Capsule size, and B. Quantification of crude capsular polysaccharide.

The above results clearly showed that capsule production was significantly up-regulated in the $\Delta cuf1$ mutant, suggesting a negative role of *CUF1* in the biosynthesis of the cryptococcal polysaccharide capsule.

2.3 Restoration of capsule production phenotype by iron in $\Delta cuf1$ mutant

Iron has been shown to be an inhibitory element of cryptococcal capsule[34]. Copper-dependent transcription factor Mac1 in *S. cerevisiae* is required for iron uptake[35]. In *C. neoformans* mutations in the copper transporter Ccc2 and the copper chaperone Atx1 result in growth defects under iron limiting conditions, indicating that copper and iron homeostasis is linked[36]. Given that low iron medium can induce capsule production, we tested whether addition of iron could suppress the capsule overproduction phenotype of the *cuf*1 mutant. As shown in Fig. 3, in the presence of 20 μmol/L $FeCl_3$, capsule production in Δ*cuf*1 indeed restored to a similar level to the wild-type and the complement strain, suggesting that Cuf1 negatively regulates capsule biosynthesis via modulating the high-affinity iron uptake pathway.

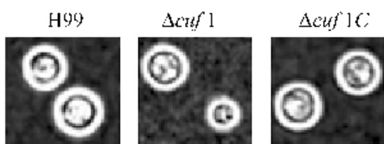

Fig. 3 Suppression of capsule overproduction in Δ*cuf*1 by high concentrations of exogenous iron. The Δ*cuf*1 mutant, in the presence of 20 μM Fe^{3+} produced similar size of capsule to that of the control strains. Other concentrations of Fe^{2+}, or Fe^{3+} had the same suppression effect (data not shown).

3 DISCUSSION

A recent study showed that disruption of the copper-dependent transcription factor gene, *CUF*1, in *C. neoformans* results in defects in growth and reduced laccase activity in low copper conditions, and drastically attenuated virulence in a mouse model[31]. In that study, the authors reported that the mutation produced a similar level of another major virulence factor, the capsule. However, we found that loss of *CUF*1 also lead to marked increased capsule biosynthesis when grown on maltose media, indicating that Cuf1 plays a negative role in capsule production. This difference in observations may be attributable to a different medium used by the report. We noticed that the authors used the malt extract agar (MEA) in stead of maltose synthetic medium for capsule production. While MEA is a suitable medium for capsule production comparing to other media, *e.g.* YPD, it may not be suitable for Δ*cuf*1 in our case due to the fact that iron might exist in malt extract that could inhibit capsule production in Δ*cuf*1. Besides, MEA still contains other carbohydrate that may affect the production of capsule.

As the major virulence factor of *C. neoformans*, capsule production is strongly induced under conditions that are highly relevant to the mammalian host environment, including iron deprivation, 5% CO_2, and mammalian serum *etc*[20, 36]. The importance of capsule size regulation is supported by the observations that capsule size varied depending on the individual organs infected during experimental murine infection and that in both the lung and the brain cryptococcal cells contained drastically enlarged capsule[18, 22]. Indeed, in mouse models, mutants with reduced capsule size displayed attenuated virulence[8,14], while mutants with increased capsule size showed hypervirulence[15]. In light of these previous findings, it is quite unexpected that the Δ*cuf*1 mutant showed markedly attenuated virulence. However, this discrepancy could be explained by the possibility that loss of Cuf1 function may affect essential biological process/processes since Cuf1 is required for copper homeostasis which is an essential trace element[31]. Another major virulence factor, laccase, required the proper function of *CUF*1. Additionally, Cuf1 is also demonstrated for the dissemination of cryptococcal cells to the brain since the Δ*cuf*1 deletion mutant was found to have reduced dissemination to the brain[31].

It has been shown that iron acquisition in *C. neoformans* involves both high- and low-affinity iron uptake pathways[38]. Capsule production in *C. neoformans* is induced under iron deprivation conditions. Our finding that the Δ*cuf*1 mutant phenotype in terms of capsule production can be converted to the wild-type with iron repletion suggests that Cuf1 may negatively regulate capsule production by modulating the high-affinity iron acquisition pathway. Additionally, our results support the view that in *C. neoformans* copper and iron homeostasis is interconnected. The control of capsule production is complex and our finding reported here should provide additional insights into how expression of this important virulence in the fungal pathogen *C. neoformans* is regulated.

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新型隐球酵母铜应答转录因子 Cuf1 与荚膜的生物合成相关性

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摘要:【目的】新型隐球酵母是人类条件致病真菌,主要感染免疫缺陷患者。该酵母最显著的特征是细胞外包被着多糖荚膜,这一重要致病因子的调控机制复杂。本文研究旨在阐述编码铜依赖转录因子的 *CUF*1 基因对其荚膜生物合成的负调控作用。【方法】以野生型菌株为对照,对 *CUF*1 缺失的突变菌株进行菌落形态观察、荚膜墨汁染色的显微观察、细胞聚沉试验以及荚膜定量分析。【结果】与野生型菌株相比,Δ*cuf*1 突变株产生的菌落更粘,显微镜下亦可明显观察到荚膜更厚。同样数量的细胞,突变株聚沉平衡后体积更大。此外,荚膜粗提物定量称重分析也证明突变株产生了更多的荚膜。并且外源铁可以回复 Δ*cuf*1 突变株荚膜过量产生的表型。【结论】铜应答转录因子 1(Cuf1)对荚膜的生物合成具有负调控作用。Cuf1 可能通过铁的高亲和吸收途径调控铁吸收而实现该作用的。

关键词: 荚膜; 新型隐球酵母; 致病因子

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