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The C-terminal of Tec1p in *Candida albicans*Ameliorate Biofilm Formation and Generation time

Walid Abu Rayyan[1]*, Nesrin Seder[2], Klaus Schroppel[3]

[1]Department of Medical Laboratory Analysis Faculty of Science, Al-Balqa Applied University, Jordan.
[2]Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmacy, Applied Science Private University, Amman, Jordan.
[3]Interfaculty Institute of Microbiology and Infection Medicine, University of T€ubingen, Germany

Abstract— The morphogenetic switch from yeast to hyphae form under the regulation of transcription regulatory genes in response to the host environment is the major contributor tothe pathogenesis of *C. albicans*. Tec1p of *C. albicans*, atranscription factor belonging to the TEA transcription factor family, harborsa conserved TEA domain for DNAbinding in the N-terminal of the transcription proteins. Tec1p is involved in mediating morphological changeby activating different signalingpathways that lead to hyphal formation. The knockout strain *tec1/tec1*is not capable of hyphae development*in vitro* and is avirulent *in vivo*. To shed the light on therelationships between the structureand function of the *C. albicans* Tec1p protein, we examined several mutant *TEC1* strains for biofilm formation and growth rate. The *TEC1* recombination cassette was constructed by fusing the *TEC1* ORFs behind a tetracycline promoter. The cassette is flanked with a homologous sequence of *ADH1* promoter to facilitate homologous recombination into the promoter region of the *ADH1* allele of the *tec1/tec1* of the CaAs12 strain.The studies of biofilm formation and growth rate of*TEC1*mutants have revealed an essential role of CHAM (C-terminal Hyphal Activation Motif) inexerting virulence in *Candida albicans*.Herewith we conclude that the activity of Tec1p cannot be executed by autonomous activation of Tec1p itself but rather by a heterodimeric complex formation with a cooperative partner which is not discovered until now. In conclusion, the C-terminal hyphal activation motif CHAM of Tec1p is essential for biofilm formation and virulence *in vitro*.

Keywords: *TEC1,* Candida albicans, Biofilm, Virulence genes, Fungi.

INTRODUCTION

The control of morphological development

The study of hyphal growth in *C. albicans* has benefited from the genetic relatedness to the brewer's yeast *S. cerevisiae*, not only because it has been largely studied but even it shares a high degree of homology in the genetic composition and signals mediating with *C. albicans*[1]. In response to the environmental factors, several signaling pathways are sensing the environmental cues and mediate the response by regulating the expression of a group of hyphae-specific genes (HSG) triggering *C. albicans* transition from yeast to pseudohyphae or hyphae forms [1].

Briefly, we summarize some of the signaling pathways involved in the morphogenetic development of *C. albicans* as shown In Figure 1.

The cAMP-PKA pathway

One of the major signaling pathways is the cAMP-PKA pathway, which includes several catalytic processes that promote morphogenesis development through the activation of a set of positive regulatory transcription factors [2]. The response regulation for environmental factors is mediated through a set of sensing genes working upstream of the cAMP-PKA pathway, first of all, the amino acid sensor Csy1p plays an important role in filamentation in response to serum or amino acid-containing medium. *csy1* mutants showed altered colony morphology and impairment of filamentous growth in serum-containing media [3]. The availability of glucose in the medium triggers the morphological transition. The glucose sensing unit consists of two G protein-coupled receptors*GPA1* and *GPA2*. The deletion of *gpa1* and *gpa2* confers a defect in hyphal formation and morphogenesis [4]. The sensed signals are transmitted through the major hyphae regulator *RAS1*, which is required for activating the cAMP-PKA pathways; *ras1* mutants have a severe defect to show hyphal development in response to glucose and serum stimulation [5]. Based on the phenotypic similarity observed in the guanine nucleotide exchange factor Cdc25 mutants, it was concluded that *Ras1- Cdc25* pathway functions upstream of the cAMP-PKA pathway. The cAMP pathway in *C. albicans* consists of three components, the adenylate cyclase protein which is only encoded in *Cdc35/Cyr1* gene, the *Srv2* adenylate cyclase-associated proteinthat regulates adenylate cyclase activity not only required for wildtype germ tube formation but also for virulence in a mouse model of systemic infection [6], and the *PDE2* gene, encoding the high-affinity phosphodiesterase which is required for suppressing the cAMP pathway [7].

The sensed signals are mediated from the cAMP pathway and activate the PKA pathway which consists of three subunits regulating the cAMP-dependent PKA encoded by the *BCY1* gene and two catalytic subunits of cAMP-dependent PKA *Tpk1/Tpk2* [8]. The protein kinases (PKA) are structurally conserved, consisting of two catalytic subunits that are inactivated by the binding of a homodimer of regulatory subunits. In such pathways, the external cues elevate intracellular levels of cAMP, which it's binding to the regulatory subunits liberates and activates the catalytic subunits. Bcy1p activates the catalytic subunits by liberating the regulatory subunit and then tethering them together. The two *Tpk* isoforms of *C. albicans* are positively regulating the hyphal formation redundantly, but each has a distinct phenotypic effect, for example, *tpk1* mutants are defective in hyphal formation on solid media but are less affected in liquid media, whereas *tpk2* mutants partially affected on solid media but are blocked in liquid medium [9].

Downstream of the cAMP-PKA pathway, the first transcription factor Efg1ptakes a place. Along with the cooperative binding of the Flo8p transcription factor, Efg1p activates the expression of the second transcription factor and the hyphal regulator Tec1pwhich is substantially implicated in mediating and acting a set of hyphae-specific genes which are required for the morphological development of *C. albicans.* Various feedback pathways regulate the level of Efg1p expression,for example, the PKA pathway activates Efg1p by

phosphorylating the threonine residue at position 206. Also,*EFG1* is an auto-regulator gene, which suppresses and controls the level of Efg1p expression by binding to the homologous DNA binding clusters in its own TATA box promoter [10]. *efg1* mutant strains share a moderate but not complete defect in hyphal growth in response to many environmental conditions. The addition of serum or GlcNAc as an inducer in liquid or solid mediums completely blocked the hyphal formation in *efg1* null mutants. In contrast to microaerophilic or embedded conditions, the hyphal formation was unaffected in the *efg1* mutants [11].

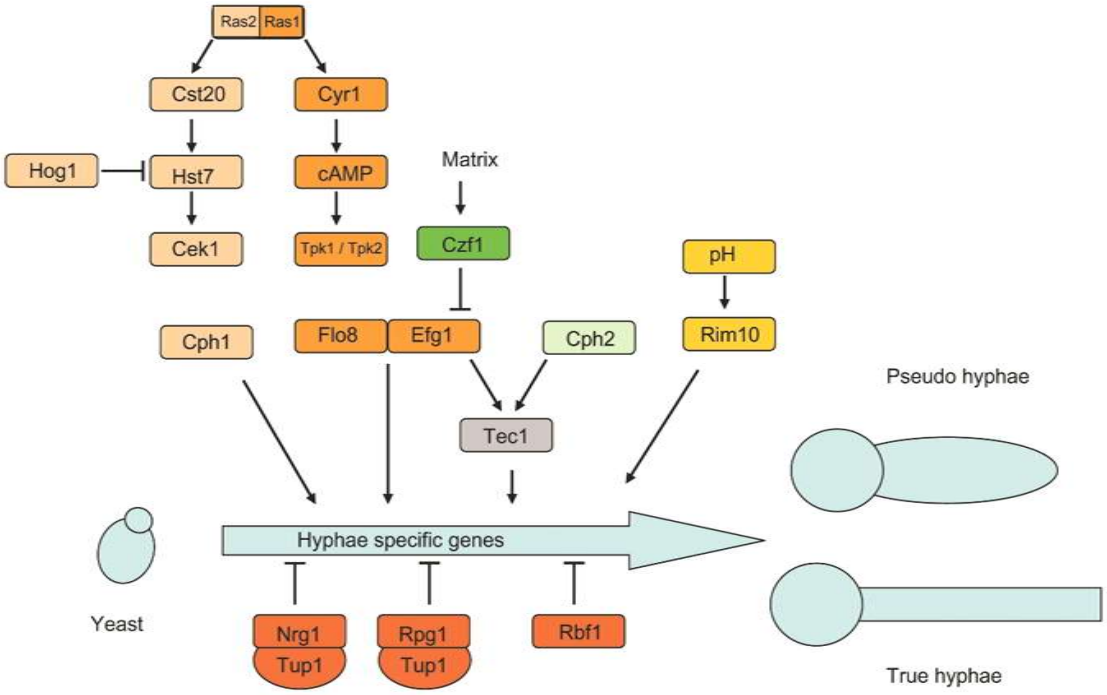

Figure 1: Multiple signaling pathways involved in hyphal transition control in *C. albicans*

Positive control: The Ras1 functions upstream of the Efg1mediated cAMP pathway and Ras2 functions upstream of the Cph1-mediated MAPK pathway. The Hog1pathway may inhibit the Hst7 in oxidative stress conditions. The czf1 pathway may inhibit Efg1 in the matrix medium. Tec1 is controlled by Cph2 and Efg1. Rim101 functions as an independent pathway.

Negative control: Hyphae-specific genes are repressed by Nrg1/Tup1 and Rpg1/Tup1 pathways. Arrows stand for activation. Bars stands for inhibition.

The role of Tec1p in the virulence and morphology of *C. albicans*

Transcription factors also called sequence-specific DNA-binding factors, are proteins that bind to specific DNA sequences, thereby controlling the transcription of genetic information from DNA to mRNA. [12]. The hyphal regulator gene *TEC1* harbors 2,322 nt (nucleotides), the N-terminal of Tec1p contains the evolutionarily conserved TEA DNA-binding domain [13] while the C terminus domain mediates gene activation [14].

Tec1p was initially identified in *S. cerevisiae* as a regulator of the expression of Ty1 transposon insertions [13].In *S. cerevisiae*, the switch from yeast form to the cylindrical hyphae form is triggered by Tec1-Ste12 complexes and the two Ste12 inhibitors proteins Dig1and Dig2. The cooperative DNA binding is established through the filamentous responsive element (TCS) of Tec1p and the pheromone responsive element (FRE) of Ste12p which are required for haploid invasive and diploid Pseudohyphae growth [14]. In addition, it has been reported that the physiological amount of Tec1p is sufficient to activate gene expression via TCS elements alone in the absence of Ste12p. Tec1p can enter the yeast nucleus without the Ste12p complex and activates the expression of a set of *FLO11* invasive growth proteins through only the N-terminal region of the TEA/ATTS DNA-binding domain of Tec1p [14]. Whereas, the C-terminal region of Tec1p harbors a C-terminal transcriptional activation domain that is required for haploid invasive growth and *STE12*-independent activation of TCS-regulated gene expression [15]. Thus, Tec1p mediates the expression of 302 target genes that constitute two distinct classes. The first class of 254 genes is regulated by Tec1p in a *STE12*-dependent manner, while the second class of 48 genes can be regulated by Tec1p independently of *Ste12*. In addition, it has been demonstrated that Tec1p regulates enriched genes that are bound by the stress transcription factors Yap6p, Nrg1p, Cin5p, Skn7p, Hsf1p, and Msn4p [16].

Saccharomyces stabilizes Tec1-Ste12 complexes degradation by the regulation of Dig1p, whose inhibitory function on Ste12 is relieved during mating by phosphorylation through the pheromone-stimulated MAPK Fus3 and Kss1 kinase. by which, Tec1p is directly phosphorylated by the MAPK Fus3 in response to pheromone, which triggers ubiquitin-mediated degradation of CDC ^{SCF24} that phosphorylates the threonine amino acid of 273 and 276 of Tec1p [17].

In *C. albicans* Tec1p is involved in hyphal development as the *tec1/tec1* mutant failed to develop hyphal transition in *vitro* and avirulent in *vivo* model [13, 18].The regulation of Tec1p expression is mediated through the Efg1 cAMP pathway [19]. In *S. cerevisiae*, *STE12*, the homolog of *CPH1* in *C. albicans*, regulates Tec1p expression in the filamentation pathway along with the Efg1 cAMP pathway, but in *C. albicans*, the bHLH transcription factor *CPH2* regulates *TEC1* expression. Cph2p is implicated in Tec1p expression by binding to the two sterols I regulatory-like elements upstream of the *TEC1* promoter [20].

Recently, Sahni et al. demonstrated that *TEC1* is placed not only downstream of the cAMP pathway but also downstream of the MAP kinase pathway and upstream of the target genes regulated by either the filamentous or the pheromone pathways giving a bridge between the two signaling pathways. Additionally, they pointed out that Tec1p regulates a set of genes, which are not components of either signal transduction pathway. [21]

In the white pheromone response, neither *CPH1* nor *CPH2*is up-regulated by pheromone, whereas *TEC1* is highly up-regulated by MAP kinase pathway, suggesting that neither *Cph1* nor *Cph2* plays a role in *TEC1* regulation in the pheromone response pathway. Tec1p mediates the induction of gene expression by a-pheromone in white cells and does so

downstream of the heterotrimeric G protein complex and MAP kinase cascade [21]. Furthermore, Tec1p is required for the pheromone response pathway in white cells but not in opaque cells.

In the pheromone response pathway, Tec1p cooperatively binds to the promoter regions of *CSH1, PBR1, RBT5, WH11, STE2, and RBT1* genes, which all contained a WPRE (White Pheromone Responsive Elements) and were all up-regulated by pheromone in white cells. Whereas, Tec1p does not bind to the promoter regions of *KAR4 or MFA1*, which contained an OPRE (Opaque Pheromone Responsive Elements) and lacked a WPRE, and also Tec1p did not bind to the promoter of *ACT1*, which lacked both an OPRE and a WPRE element which demonstrates the dual role of Tec1p in mediating signals in the white but not in the opaque pheromone response [21].

In filamentous growth, Tec1p is a putative positive regulator for 13 genes whose concentrations are dramatically increased during hyphal formation, they contain preserved *TEC1* consequence sequences (TCS) upstream of the promoter region [22]. The expression of Tec1p regulates the expression of four distinct protein families which encode the cell wall proteins Ece1p, Cht2p, Hwp1p, Als8p, and Hyr1p, the adhesion protein Als8p, the G1 cycling Hgc1p which is required for hyphal development, the aspartyl secretary proteins Sap4-6p, and the cell polarization protein Cdc24p, Bem2p and Rho3p [3]. Furthermore, Tec1p is implicated in the direct expression of the biofilm formation regulator Bcr1p, as the promoter region harbors numerous copies of TCS sequences, Bcr1p, in turn, regulates the expression of the hyphae specific genes *ECE1, CHT2, HYR1,HWP1, RBT5, ALS1* and *ALS3* [23], which mediates the cytoskeleton and transcription development in *C. albicans*.

MATERIAL AND METHODS

Microorganisms

The Microorganisms used in this study are arranged in Table 1 according to the species type.

Table 1:*C. albicans* strains used in the study

Strain	Origin	Relevant genotype	Reference/source
SC5314		Wild type strain	Gillum et al. 1984
CaAS12	SC5314	*tec1*::hisG / *tec1*Δ::hisG-*URA3*-hisG	Schweizer, 2000
CWR01	CaAs12	*ADH1/adh1* :: pTet*TEC1*Δ (1440-1765)	Rayyan et al., 2019
CWR02	CaAs12	*ADH1/adh1* :: pTet*TEC1*Δ (2217-2232)	Rayyan et al., 2019
CWR03	CaAs12	*ADH1/adh1* :: pTet*TEC1*Δ (648-	Rayyan et al.,

		733)	2019
CWR04	CaAs12	*ADH1/adh1* :: pTet*TEC1*Δ (162-636)	Rayyan et al., 2019
CWR05	CaAs12	*ADH1/adh1* :: pTet*TEC1*Δ (1922-2232)	Rayyan et al., 2019
CWR06	CaAs12	*ADH1/adh1* :: pTet*TEC1*	Rayyan et al., 2019
CWR09	CaAs12	*ADH1/adh1* :: pTet*TEC1*Δ (1911-2232)	Rayyan et al., 2019
CWR11	CaAs12	*ADH1/adh1* :: pTet*TEC1*Δ (2019-2232)	Rayyan et al., 2019
CWR12	CaAs12	*ADH1/adh1* :: pTet*TEC1*Δ (2127-2232)	Rayyan et al., 2019

Strains culture

The strains and growth conditions

The yeast strains of *C. albicans* used in this study are listed in Table 1.The strains wereconserved as proposed by[24] and stored in the form of frozen stocks in 35% glycerol at –80°C. Strains were cultured and grown in YPD medium at 25 °C, with vigorous shaking at 150 rpm. To solidify the plates 1.5% of agar was added to the broth. 200 µg/ml of nourseothricin was used in combination with YPD medium after being autoclaved and cooled to 50°C. Strains were also propagatedon synthetic (CM-URA) agar plates containing a complete supplement medium withouturacil.

E.coli strains used in this study were grown in LB (Lauria-Bertiani) medium at 37 °C for 24hrs at constant shaking at 200 rpm. 100 µg/ml of Ampicillin was added to the medium for the selection or t propagation of resistant strains when needed.

For morphological development experiments, both liquid and solid mediums were used. Strains were inoculated in liquid DMEM (Dulbecco´s Eagle Medium) containing 40 mM HEPES buffer 5% Co_2 in the presence or the absence of Tet at 25°C for 24 hrs. For invasive growth, cells were spotted on spider medium in the presence or the absence of Tet at 37°C for 5 days.

For EUCAST test strains were grown on sabouraud (Sab) plates supplemented with 40 µg/ml gentamicin for 16hrs at 37°C.

Biofilm development in *TEC1* mutant strains

Strains were pregrown in YPD medium under shaking at 37°C for 16 hrs. Afterward, 5 ml of cell suspension was pelleted and resuspended in 2 ml of phosphate buffer solution (PBS). The

cells were counted with a Neubauer chamber. The silicon sheets were cut into 1,69cm² squares and pretreated after sterilization with fatal bovine serum (FBS) with gentle shaking at 37°C for 16 hrs. Later, the cells were suspended in PBS at a concentration of 10^7 cells per ml. 4 ml of the suspension was incubated at 37°C for 1.5 h without shaking in a 12 wells plate containing pretreated silicon sheets, this represents the adherence phase. Afterward, the silicone sheets were transferred into new 12 wells plates containing 4 ml of fresh RPMI 1640 medium supplemented with or without doxycycline (50µg/ml). The plates were incubated for 15 h, 39 h, and 63 h with shaking at 37°C. After incubation, biofilms grown on silicone sheets were scraped off into fresh 12 wells plates containing 4 ml of PBS. The well's contents were removed with a 15 ml pipette and filtrated. Filters (Sartorius Stedim) were weighed before the filtration step to measure the empty weight of each filter.

Filters were dried at 37°C for 24h after the filtration step. The biofilm dry weight was determined by subtracting the empty weight of the filters from the filter weight after filtration. Pictures of silicone sheets were taken after 63 h of incubation and a short time of drying.

RESULTS

Biofilm formation of the revertant strains

It was demonstrated that Tec1p mediates biofilm formation by regulating the expression of *C. albicans* biofilm regulation gene *BCR1*, which works downstream of *TEC1*, We addressed the question of whether the mutation of *TEC1* can affect the biofilm formation capability of the revertant strains.

To analyze the mutants in terms of biofilm formation, we cultivated the strains CWR03, CWR05, and CWR06 along with control strains SC5314 and CaAS12 in the absence or presence of Tet. The full-length construct in CWR06 was able to develop a heavy biofilm mass to the *tec1/tec1* mutant background after Tet-induced gene expression, similar to the biofilm mass formed by the wildtype SC5314 strain. Whereas, in the absence of Tet, the biofilm mass was as low as the biofilm mass formed by CaAS12 as shown in Figure 2.

The CWR03 strain which has a deletion in the TEA domain formed the lowest biofilm mass in both conditions of the presence or the absence of Tet Figure 3. This indicates that the driven activity of Tec1p is substantially abrogated by the defect of DNA binding through the TEA domain.

The C-terminal mutant CWR05 strain was not able also to restore the biofilm formation capability in comparison with the CWR06 strain, rather the biofilm mass was similar to the biofilm mass of the knockout CaAS12 strain. This indicates that the driven activity of Tec1p expression is also mediated through the C-terminus of Tec1p.

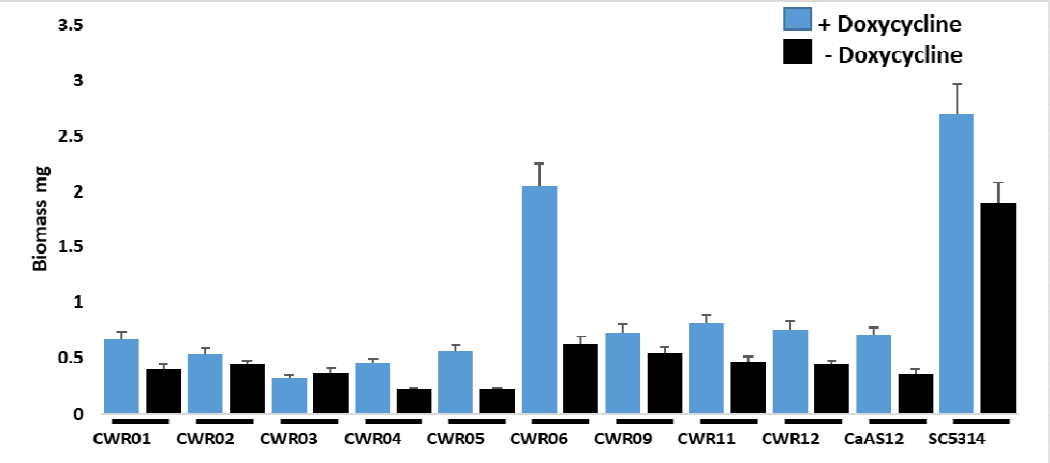

Figure2: Biofilm mass of the revertant strains

Biomass (dry weight) of biofilms after 63h growth at 37°C in RPMI medium with or without doxycycline (50µg per ml). White bars: without doxycycline. The wildtype has the highest biomass. The full *TEC1* revertant CWR06 produced similar biomass of SC5314 with Tet. Both strains CWR03 and CWR05 were able to form biomass almost as the CaAS12 *tec1/tec1* strain.

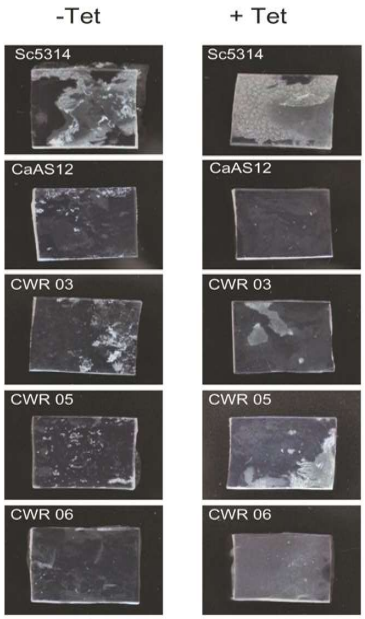

Figure3: Biofilm formation of revertant strains on silicone sheets

Silicon sheets after incubation and a short time of drying after 63h growth at 37°C in RPMI medium with or without doxycycline (50µg per ml). The wildtype showed heavy biofilm structures in the presence or absence of Tet. The full *TEC1* revertant CWR06 showed a heavy biofilm structure in the presence of Tet. Both strains CWR03 and CWR05 showed low biofilm structures similar to the CaAS12 *tec1/tec1* strain in the presence or absence of Tet.

Growth analysis of the revertant strains

To elucidate the effect of constitutive Tec1p expression on the growth rate for the *TEC1* mutant strains, we propagated the revertant strains CWR01, CWR02, CWR03, CWR04, CWR05, CWR06, CWR09, CWR11, and CWR12 in addition to the wildtype SC5314 and the knockout strain CaAS12 in YPD medium at 25°C in the presence or absence of tetracycline, and the optical density was measured in regular intervals Figure 4.

The growth rates of the revertant strain as well as the wildtype SC5314 were affected by the addition of doxycycline as the optical density readings were less than the readings for the same strains in the medium without doxycycline. The knockout CaAS12 strain readings were almost similar in both conditions which points out that the possible effect of doxycycline is mediated in one way or another through hyphal augmentation for both wildtype SC5314 and revertant strains.

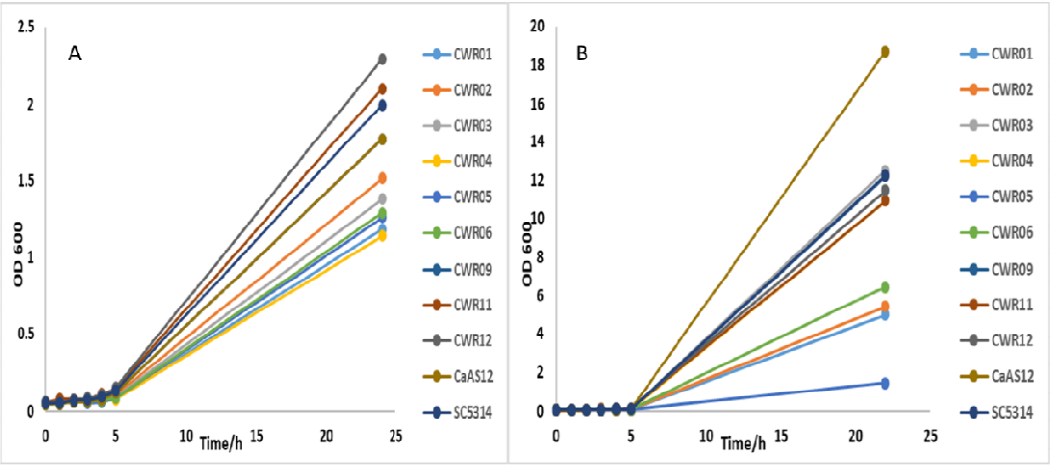

Figure4: The growth curve for the revertant cells. A) Growth curve without tetracycline B)Growth curve with tetracycline

Revertant strains along with SC5314 and CaAS12 strains were grown in YPD medium for 24 hrs at 25°C. The CWR05 strain had the lowest growth rate in collection whereas the CWR02 strain had the highest growth rate. Revertant strains along with SC5314 and CaAS12 strains were grown in YPD for 24 hrs at 25°C in the presence of doxycycline. The knockout strain CaAS12 had the highest growth rate in the addition of tetracycline.Whereas, the CWR05 strain had the lowest growth rate in the presence or the absence of doxycycline.

DISCUSSION

Biofilm formation genes are regulated by the CHAM motif of Tec1p

The biofilm activator Bcr1p protein harbors several TCS binding sequences in the promoter region. This demonstrates a direct binding of the TEA domain of Tec1p on the homologous sequence in the promoter region of Bcr1p. It is established that transcription factors comprise two functional regions in their structures. The microorganisms develop biofilm forms to establish a shield against immune defense [25, 26] Mostly the N-terminal is required for the binding of a homologous sequence to the DNA complex, and the C-terminal harbors the gene-activating domain. To prove the concept and to give evidence on the importance of the C-terminal of Tec1p in mediating genes activation, we performed the biofilm formation studies using the strains CWR03, CWR05, and CWR06 as well as the control strains SC5314 and CaAS12. As it is anticipated the TEA binding domain deletion in the CWR03 strain influenced the biofilm formation as the biofilm mass was similar to the mass formed by the knockout *tec1/tec1* CaAS12 strain. This indicates the crucial effect of the TEA domain in the cooperative transcription factor – DNA binding. The DNA binding studies by Sehnal [27] have revealed abolish of DNA binding in the EMSA studies when the TEA domain is absent. Similar results were reported by Nobile et al, as the loss of the *TEC1* gene has inquired no activation of Bcr1p [11]. They reported also that the *tec1/tec1*mutant grew largely as free-living cells in biofilm-formation assay, yielding a relatively translucent substrate and turbid medium. Thus the biofilm produced by the *tec1/tec1* strain has less biofilm mass and it was composed exclusively of yeast cells. In contrast, the *TEC1/TEC1* reference strain has dramatically increased the expression of Bcr1p and produced a heavy biofilm mass that included many hyphal filaments [11]. The expression of the full-length Tec1p in the revertant

CWR06 strain produced almost a similar biofilm mass as the wildtype SC5413 which indicates that ectopic expression of *TEC1* can restore the biofilm formation ability and mediate the expression of biofilm formation genes through activation of the expression of *BCR1* by direct binding to the TCS elements in the promoter region. This also proposed that there is no phosphorylation process required for *TEC1*-driven activity as there was no activation for the c-AMP pathway. On the other hand, the weight of biofilm mass formed by the mutant strains CWR04 and CWR05 were similar to the biofilm mass of the knock out CaAS12 strain, which indicates not only the glutamine rich regions in both of N and C terminal domains are importance for the activation of *BCR1* expression but even this gives a second evidence of the involvement of C-terminal domain of Tec1p in gene expression activity. The DNA binding studies of Tec1p have revealed the importance of the glutamine N terminal region in DNA binding as mutation deletion in this region has revealed a loss of DNA binding in both transcription factors *TEC1* and *TEF1*[27, 28].

The growth rate is mediated by Tec1p

During the evagination process of *C. albicans*, the cyclin-dependent kinases family plays a role in controlling different stages of meioses which regulate the generation time by collaboration with feedback pathways.

In *C. albicans,* mutational analysis studies have revealed a feedback mechanism between Tec1p and cyclin-dependent kinases. The levels of Tec1p are dramatically increased in serum addition conditions which also elevate the levels of CDC24p, as do the G-protein *RHO3* and the Rho1 GTPase activating protein *BEM2* transcript levels. These results suggest that a positive feedback loop between Cdc24p and Tec1p contributes to locating Cdc42p at the tip of the germ tube [29]. In our growth studies, there was no deleterious effect of *TEC1* deletion on the *Candida* strain's growth; rather it affected the morphology and the virulence. The rates of growth for the revertant strainsCWR01-06, CWR09, CWR11, and CWR12, the wildtype SC5423, and the knockout CaAS12 were relevant in the conditions without tetracycline. Whereas in the condition when doxycycline was added, the strains behave differently, the knockout CaAS12 growth rate was not affected by the addition or the absence of Tet. The CWR03 strains had the highest growth rate after CaAS12 then the wildtype SC5314. The CWR01, CWR02, and CWR06 strains, which harbor a functional copy of Tec1p showed the lowest growth rate when tetracycline was added. This may be contributed to the effect of a low optical density measurement duo to either the growth in a hyphal form in the CWR01, CWR02, and CWR06 strains has affected the optical density measurement (the measurements were conducted for three distinctive times), or the possibility of the presence of a negative feedback mechanism mediated through Tec1p to activate meiosis arrest for establishing the anabolism of hyphae specific proteins or even the growth in hyphal form requires more generation time. Interestingly, the knockout strain and the CWR03 had the highest growth rate in the presence of Tet this indicates that Tec1p is highly implicated in mediating signaling events controlling the generation time. But surprisingly, the CWR05 strain showed a lost growth rate in both incubation conditions. This result could not be explained from the perspective of the structure-function relationship.

CONCLUSION

We conclude that a C-terminal hyphal activation motif CHAM of Tec1p is essential for biofilm formation and growth rate *in vitro*.

CONFLICT OF INTEREST

The authors declare no conflict of interest

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