Poisoning a fungal pathogen by D-amino acids

A thesis submitted in partial fulfillment for the degree of

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by

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“The woods are lovely, dark and deep,

But I have promises to keep,

And miles to go before I sleep,

And miles to go before I sleep.”

-Robert Frost

(Stopping by woods on a snowy evening)

DECLARATION

I hereby declare that the work described in this thesis entitled "**Poisoning a fungal pathogen by D-amino acids**" has originally been carried out by me under the supervision of Prof. Kaustuv Sanyal, Professor, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advance Scientific Research, Jakkur, Bangalore 560-064, India.

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Date: 12.4.2019

CERTIFICATE

This is to certify that this thesis entitled "**Poisoning a fungal pathogen by D-amino acids**" submitted by Kuladeep Das for the partial fulfillment of the degree of Masters of Science, as part of Integrated Ph.D. program at Jawaharlal Nehru Centre for Advance Scientific Research, was based on the studies carried out by him under my supervision.

Kaustuv Sanyal
15/04/2019

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Kuladeep

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List of Abbreviations

aaRS	aminoacyl-tRNA synthetase
bp	base pair
DTD2	the gene corresponding to the orf19.297 in *Candida albicans* SC5314 strain
*DTD2H**	the gene corresponding to the orf19.279 in Candida albicans SC5324 strain
D-aa	Dextrorotatory amino acid
DNA	Deoxyribonucleic acid
DTD	D-aminoacyl-tRNA deacylase
EF-Tu	Elongation factor thermo unstable
hr	hour
L-aa	Levorotatory amino acid
min	minute
µL	microlitre
µM	micromolar
ORF	open reading frame
RNA	ribonucleic acid
sec	second
tRNA	transfer RNA

Other abbreviations used in this study:

1. For designating the standard amino acids, the one and three letter codes have been used interchangeably throughout the text.

* - The '*DTD2H'* nomenclature doesn't correspond to the standard nomenclature in the Candida Genome Database. The orf19.297 has been indicated as *DTD2H* or Ca*DTD2H* in this study for simplicity.

1. Introduction

1.1 Overview

Most of the biological processes in all living systems are carried out by proteins. These are long polymers constructed during ribosomal translation as linear polypeptide chains by joining together amino acids by peptide bonds, whose number and order is dictated by triplet genetic code (Crick, Barnett et al. 1961). There are 20 'proteogenic' amino acids (excluding selenocysteine and pyrrolysine) used by all organisms for protein biosynthesis. Except for glycine, each amino acid is "chiral" – existing in both **D**extrorotatory (D) and **L**evorotatory (L) form (**Figure 1**). During ribosomal translation, only L-amino acids and glycine are accepted and D-amino acids are prevented from being incorporated into the growing polypeptide chains (Banik and Nandi 2013). This 'homochirality' in proteins is maintained and seems to be common to all life forms on earth.

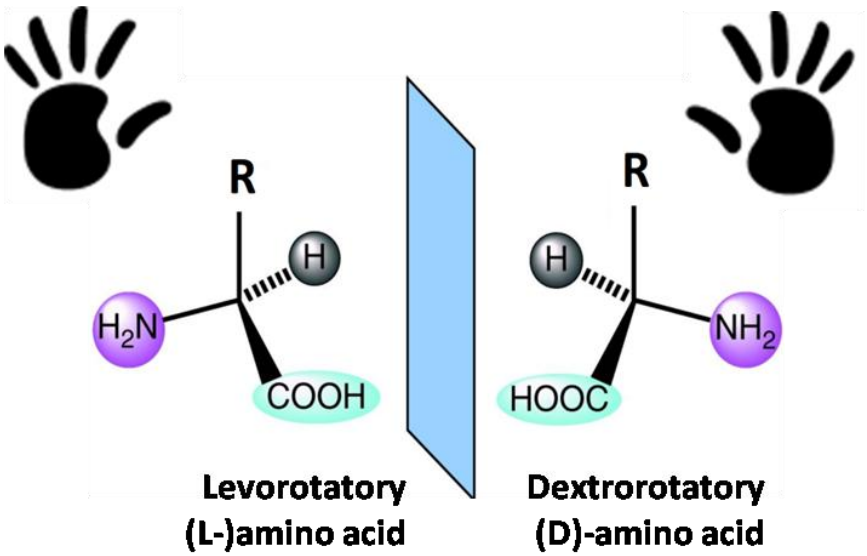


Figure 1. Amino acids are chiral molecules. Except for glycine, every other amino acid is "chiral"-existing in both Dextrorotatory (D) - and Levorotatory (L)-form. During translation, D-enantiomers are prevented from being incorporated into growing polypeptides thereby maintaining protein homochirality. Image modified from (Blackmond 2010).

1.2 Free D-amino acids are commonly found in nature

While D-amino acids find no place during protein translation and organisms mostly synthesize L-enantiomers, the sources and occurrence of D-amino acids are not so uncommon. Firstly, due to their intrinsic chemical features, the amino acids can undergo acid- and base-catalyzed racemization through keto/enol tautomerism and generate D-amino acids in nature (Martinez-Rodriguez, Martinez-Gomez et al. 2010). This spontaneous chemical racemization is favored at the earth's surface temperatures and pH (Fujii and Saito 2004). Thus the appearance of D-enantiomers is inevitable, and natural habitats like oceans, soils, and sediments are enriched in many D-amino acids (Zhang and Sun 2014). Secondly, L- to D-racemization in many organisms is enzymatically catalyzed by racemases (Yoshimura and Esak 2003) and the resultant D-amino acids are incorporated into non-protein molecules. The most common example is the peptidoglycans in bacterial and cyanobacterial cell walls which contain D-alanine, D-glutamic acid, and occasionally D-aspartic acid (Rogers 1974). Other examples include peptide antibiotics (Martinez-Rodriguez, Martinez-Gomez et al. 2010), peptide siderophore molecules (Drechsel and Jung 1998), and surfactins (Peypoux, Bonmatin et al. 1999), etc. In higher eukaryotic organisms including animals and humans, D-amino acids are known to fulfill specific biological functions (Fuchs, Berger et al. 2005) . Certain D-amino acids like D-serine and D-glutamate, produced endogenously in the central nervous system play an important role in learning and memory (Riedel, Platt et al. 2003, Henneberger, Papouin et al. 2010). D-aspartate is crucial for its role during development and endocrine function (Fuchs, Berger et al. 2005).

1.3 Restricting D-amino acid incorporation into proteins

While D-amino acids seem to be ubiquitously present and equally necessary as discussed above, their incorporation into proteins is harmful. The diverse array of functions carried out by proteins depend on their proper folding which is again determined in part by the sequence and chirality of amino acid monomers in the polypeptide chain. Most D-amino acids are strong helix destabilizers and hence, the entry of D-enantiomers into protein would render it functionally defective due to misfolding (Krause, Bienert et al. 2000).

Organisms employ several mechanisms to restrict D-amino acid's entry into a translated protein (Wydau, van der Rest et al. 2009). One general strategy is to maintain the intracellular D-amino acids' level significantly lower than their L-counterpart. Prokaryotes have a D-amino acid

dehydrogenase enzyme that catalyzes the oxidation of D-amino acids into their corresponding oxo-acids (Olsiewski, Kaczorowski et al. 1980, Ingledew and Poole 1984). Several eukaryotes possess D-amino acid oxidases that control endogenous D-amino acids (Pollegioni, Piubelli et al. 2007). In organisms belonging to the fungal kingdom, D-amino acid acylase is found (Zenk and Schmitt 1965, Bhatt, Yogavel et al. 2010). Several other enzymes with narrow specificity such as D-serine deaminase, alanine-, and serine-racemases, *etc* also play an important role to maintain the disequilibrium against D-amino acids (Korte-Berwanger, Sakinc et al. 2013, Wei, Qiu et al. 2016).

The ribosomal protein biosynthesis requires that an amino acid is charged with its cognate transfer RNA molecule (tRNA), a step is also known as tRNA-charging. This step is catalyzed by the enzymes amino-acyl tRNA synthetases (aaRS) in an ATP-dependent manner (Woese, Olsen et al. 2000). The product of this reaction is an aminoacyl-tRNA complex, which is then escorted to ribosomal A-site by Elongation factor-Tu (EF-Tu) during translational elongation. Several checkpoints of the translational machinery work in tandem to restrict D-amino acids into protein translation. This includes aaRSs, EF-Tu and ribosome, all of which are known to (weekly) discriminate the chirality of the amino acids (Jonak, Smrt et al. 1980, Bhuta, Quiggle et al. 1981, Agmon, Amit et al. 2004, Englander, Avins et al. 2015). However, this discrimination is not 100% committed, and significant D-aminoacylation of t-RNAs by various aminoacyl-tRNA synthetases including tyrosyl-, tryptophanyl-, aspartyl-, lysyl- and histidyl-tRNA synthetases have been detected both *in vitro* as well as *in vivo*(Calendar and Berg 1967, Soutourina, Blanquet et al. 2000, Soutourina, Soutourina et al. 2004, Takayama, Ogawa et al. 2005, Sheoran, Sharma et al. 2008).

The formation of a D-aminoacyl-tRNA complex inside the cell is itself detrimental in two ways- first, a high probability of incorporation into growing polypeptide chain could lead to global misfolding and second, these metabolically inactive products could get accumulated in the cell leading to depletion of free cellular tRNA pool, which would indirectly affect the rate of translation (Ahmad, Routh et al. 2013). A freestanding enzyme D-aminoacyl-tRNA deacylase (DTD) is able to discriminate the incorrect D-aminoacyl-tRNA from correct L-aminoacyl-tRNA complex and catalyzes their hydrolysis to free the tRNA from the D-amino acids (Calendar and Berg 1967, Wydau, van der Rest et al. 2009, Zheng, Liu et al. 2009).

1.4 D-aminoacyl-tRNA Deacylase-a chiral proofreading enzyme

Functionally, D-aminoacyl-tRNA deacylase, or DTD, belongs to a family of hydrolase enzyme capable of editing in *trans* mis-aminoacylated tRNA's (Wydau, van der Rest et al. 2009). This family also includes several freestanding factors homologous to aminoacyl-tRNA synthetase editing domains such as AlaXs, peptidyl-tRNA hydrolase (Menninger 1976), etc. DTD can distinguish the mischarge D-aminoacyl-tRNA from the cognate L-aminoacyl-tRNA counterpart. The enzyme shows a broad specificity, thus, being able to hydrolyze a number of D-aminoacyl moieties attached to a tRNA molecule (Calendar and Berg 1967, Soutourina, Blanquet et al. 2000, Soutourina, Soutourina et al. 2004). Given its strategically important role, the DTD function is conserved in all three domains of life and three distinct types of DTDs have been reported. The canonical DTD (also known as DTD1) is commonly found in bacteria and eukaryotes and shows a high degree of sequence conservation from bacteria, yeast to human (Soutourina, Plateau et al. 1999, Soutourina, Blanquet et al. 2000, Zheng, Liu et al. 2009). For instance, the sequence identity between the DTD from *E. coli* and humans is 39%. The second variant of DTD, termed as DTD2, structurally unrelated to the canonical DTD is found in archeae and plants (Rigden 2004, Ferri-Fioni, Fromant et al. 2006, Wydau, Ferri-Fioni et al. 2007). Additionally, a DTD-like domain appended to the N-terminus of Threonyl-tRNA ligase has been found in many archaeal species and their D-aminoacyl deacylase activity has been experimentally validated (Rigden 2004, Hussain, Kruparani et al. 2006). DTD3, a third type of DTD, is found in many cyanobacterial species whose genome has been sequenced (Wydau, van der Rest et al. 2009). An overview of the three distinct *DTDs* is shown in **Figure 2**.

Mechanistically, the DTD enzyme faces two challenges- a) to reject all L-aminoacyl-tRNA complexes, but b) to act on multiple D-aminoacyl-tRNA complexes. This dual identification process is known as 'chiral proofreading', a term coined in 2013 (Ahmad, Routh et al. 2013). In the past decade, the crystal structure of DTD from several organisms has provided an in-depth structural basis for DTD's enantioselectivity. Since first crystal structure of *E. coli*Dtd reported in 2001 identified the novel DTD-like fold (PBD id: **1JKE)** (Ferri-Fioni, Schmitt et al. 2001), the Dtd protein structure from several organisms including *Haemophilus influenzae* (PDB id: **1J7G**) (Lim, Tempczyk et al. 2003), hyperthermophilic bacterium *Aquifex aeolicus* (PBD id: **2DBO**) and *Homo sapiens* (PBD id: **2OKV**)(Kemp, Bae et al. 2007) has been solved. The functional DTD holo-enzyme is a homo-dimer with two active sites situated at the

dimeric interface (Ahmad, Routh et al. 2013). An invariant, cross-subunit Gly-*cis*Pro motif, conserved across all organisms looked into, is crucial for capturing the chiral center of D-aminoacyl-tRNA(Ahmad, Routh et al. 2013).

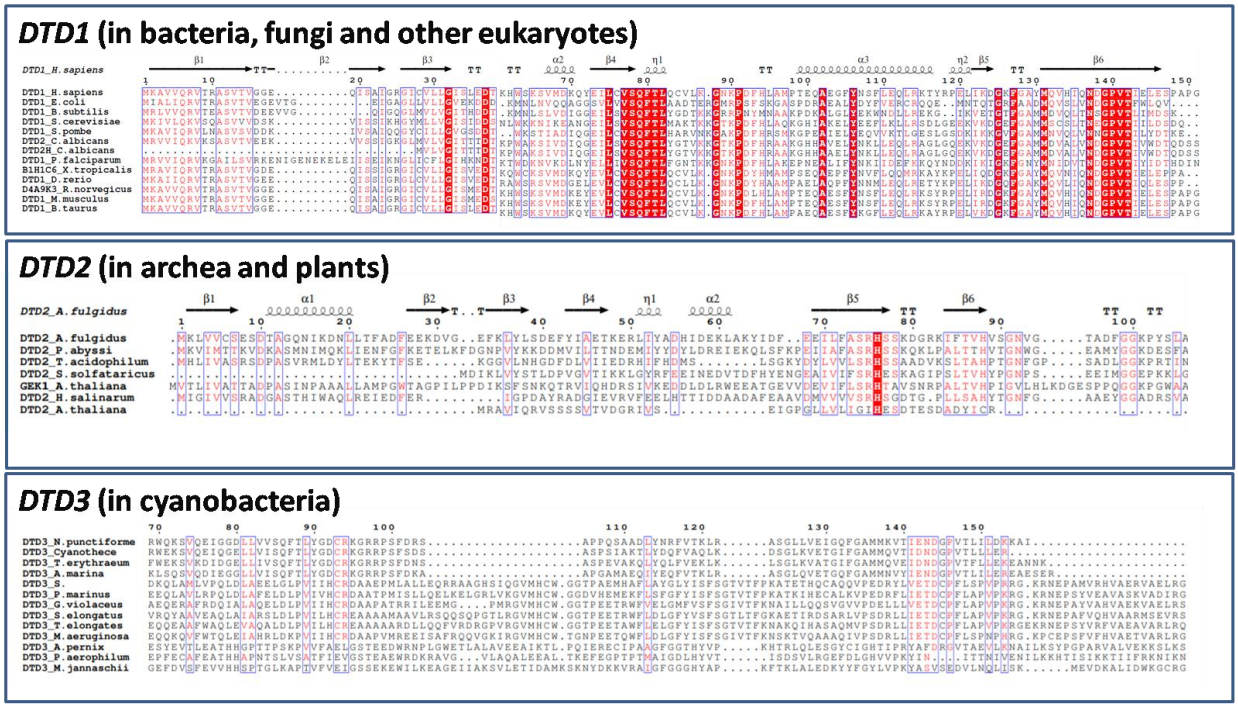


Figure 2. Multiple sequence alignment showing the three distinct types of DTD enzymes from different domains of life. While DTD1, commonly found in bacteria and eukaryotes show high sequence identity, the DTD2 (found in archeae and plants) and DTD3 (found in cyanobacterial) display poor sequence conservation. (Sequences were retrieved from Swiss-Prot database).

At the mechanistic level, this enzyme acts on multiple D-aminoacyl-tRNA complexes by identifying unique and common features from both D-amino acid as well as tRNAs, and actively reject L-counterpart from getting hydrolyzed (Ahmad, Routh et al. 2013, Routh, Pawar et al. 2016). Since glycine is achiral, the glycyl-tRNA^{Gly} complex is not actively rejected from DTD active site and hence prone to be hydrolyzed or 'mis-edited', a consequence of DTD-mediated mechanism. At the cellular scenario, this cognate product is prevented from getting hydrolyzed by the DTD enzyme by EF-Tu mediated protection (Routh, Pawar et al. 2016) as well as due to a unique discriminator base in tRNA^{Gly} (Kuncha, Suma et al. 2018). A positive consequence of this DTD-design error comes as a plus point while dealing with mis-acylated glycyl-tRNA^{Ala} complexes as explained below.

The side chains of three amino acids- glycine, L-alanine, and L-serine are sterically similar. An alanyl-tRNA synthetase, which charges L-alanine to its cognate $tRNA^{Ala}$ molecule also frequently mischarges glycine and L-serine, 1 in 240 and 1 in 500 times, respectively (Tsui and Fersht 1981), a rate much higher than the overall error rate of ~10^{-4}-10^{-3} during protein translation (Ogle and Ramakrishnan 2005). Several trans-editing factors, collectively referred to as AlaXs, which are free-standing homologs of the AlaRS cis-editing domain, act as cellular checkpoints to breakdown L-Ser-$tRNA^{Ala}$ complexes (Guo, Chong et al. 2009). However, no such trans-editing factors are known to act against mis-charged Gly-$tRNA^{Ala}$ complex. This product, which is not L-aminoacyl-tRNA, is recognized by the DTD enzyme which catalyzes the hydrolysis of the ester bond thereby preventing alanine to glycine mistranslation (Pawar, Suma et al. 2017). Thus, DTD acts beyond its conventional chiral proofreading role to prevent glycine mischarging by the AlaRS enzyme. These two well-understood roles of DTD enzyme are summarized in **Figure 3**.

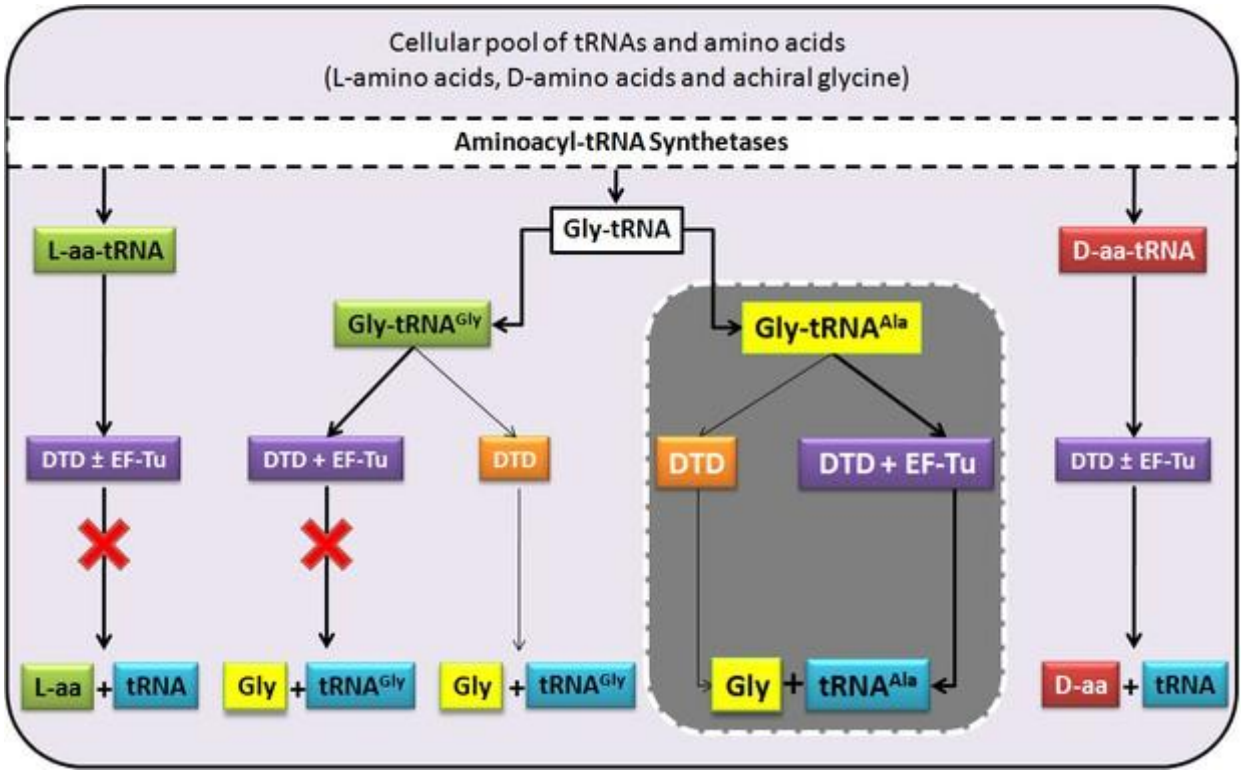


Figure 3. DTD decouples D-amino acids and mischarged Gly-$tRNA^{Ala}$. Inside a cell, aminoacyl-tRNA synthetases charge amino acids with their cognate tRNAs. DTD decouples D-aminoacyl-tRNA (D-aa-tRNA) complexes (extreme right), without acting on L-aa-tRNAs (extreme right). Since glycine is achiral, Gly-$tRNA^{Gly}$or mischarged Gly-$tRNA^{Ala}$are acted upon by DTD. In a cellular context, indicated by bold arrows, EF-Tu protects Gly-$tRNA^{Gl}$ from DTD but not the Glu-$tRNA^{Ala}$ complex. (Adapted from (Pawar, Suma et al. 2017).

1.5 Regulation of individual vs. community-dwelling behavior of microorganisms

As pointed out above, D-amino acids are non-candidate for protein biosynthesis. However, these form crucial components in many specialized structures including peptidoglycans, non-proteogenic peptides, etc. A recently understood phenomenon of some D-amino acids in modulating the individual vs. community-dwelling behavior of microorganisms has surfaced. Most bacteria are known to form biofilms, an organized community of cells enclosed in a self-produced extracellular matrix, thereby protecting them from environmental insults (Karatan and Watnick 2009). However, as biofilms age, factors like limiting nutrients, accumulation of waste products are detrimental to the cells, and hence it is advantageous for the cells to dissociate from aging biofilms. This dissociation is triggered by biofilm-disassembly, mediated by D-amino acids (Kolodkin-Gal, Romero et al. 2010). Prior to biofilm maturation, cells start producing D-amino acids such as D-leucine, D-methionine, D-tyrosine, and D-tryptophan which cause the release of amyloid fibers that linked cells in the biofilm together. Just like bacteria, diverse yeasts can form biofilms or biofilm-like structures. Some common pathogenic yeast species include *Candida albicans, Candida dubliniensis, Candida tropicalis, Cryptococcus neoformans, Aspergillus fumigatus, etc* (Desai, Mitchell et al. 2014). The central significance of biofilms related to infection biology arise due to the fact that many of these species form biofilms on implanted medical devices like catheters, endotracheal tubes, cardiac devices, urinary catheters, breast implants, *etc* (Banerjee, Gupta et al. 1997, Raad 1998, Ramage, Vande Walle et al. 2001, Al-Fattani and Douglas 2006, Martinez and Casadevall 2007, Ramage, Rajendran et al. 2011, Desai, Mitchell et al. 2014). These biofilms are recalcitrant to drug treatment due to limited drug susceptibility and hence difficult to treat.(Desai, Mitchell et al. 2014).

While fungal biofilms are structurally different from bacterial ones, there underlie certain common themes. Cells in all kinds of biofilms must be able to adhere, secrete extracellular matrix components and communicate to make resilient biofilms(Armitage 2004). Now, D-amino acids are known to be a triggering factor for disassembly of aging bacterial biofilms (Kolodkin-Gal, Romero et al. 2010). It is not known whether D-amino acids can modulate similar behavior in fungal biofilms also.

1.6 The rationale of this study

The D-aminoacyl-tRNA deacylase (DTD) are evolutionarily conserved in all three kingdoms of life (Wydau, van der Rest et al. 2009). While molecular mechanisms are relatively well-established, *in vivo* effects of loss of DTD function is less explored in most organisms except few studies (Soutourina, Plateau et al. 1999, Soutourina, Blanquet et al. 2000, Soutourina, Soutourina et al. 2004, Zheng, Liu et al. 2009). Secondly, the modulatory behavior of D-amino acids in bacterial biofilms makes it relevant to take an approach to study DTD function, biofilms and the modulatory roles of D-amino acids *in vivo*, and how free-living and pathogenic microbes are able to protect themselves against D-amino acid poisoning.

The aim of this study was to understand the role of DTD in an opportunistic fungal pathogen *Candida albicans* that undergoes phenotypic transitions between different morphological forms (Mallick, Bergeron et al. 2016, Rai, Singha et al. 2018). *Candida albicans* cells grow as single planktonic cells in the flask or in the host's body but often form a thick multilayered drug-resistant biofilm structure when the cells come in contact with a biotic or abiotic surface (Lohse, Gulati et al. 2018). We took a genetic approach to compare the D-amino acid-mediated toxicity *in Saccharomyces cerevisiae*, a free-living organism, and *Candida albicans*, an opportunistic pathogen, under conditions of loss of DTD function. We created a null mutant of *dtd* in *S .cerevisiae* and *C. albicans* in this study. Our preliminary results suggest that D-amino acids do not prevent planktonic growth of *C. albicans* unlike *S. cerevisiae* mutants, but prevent biofilm growth.

2. Results

2.1 The genetic architecture of *DTD* genes in *C. albicans*

Comparative sequence analysis using the BLAST program revealed the presence in *C. albicans* of two open reading frames (ORF's) - orf19.297 and orf1.279, encoding putative *DTD* genes homologous to the *S. cerevisiae* YDL219W encoding the D-aminoacyl-tRNA deacylase (Sc*DTD1*) (**Figure 4A**). In *C. albicans* 3rd chromosome, the two ORFs belong to a duplicated DNA segment (approx. 3.2 kb) present as inverted repeats approximated 11.3 kb apart (**Figure 4B**). While Orf19.297 encodes a full-length putative DTD protein, the other orf19.279 probably encodes a truncated version of the protein with 27 N-terminal residues missing (**Figure 4C**). The two putative ORFs have non-synonymous variations at two residues – **G70D** and **V114A** as well as two additional synonymous variations at the DNA sequence level, thus uniquely mapping long RNA reads possible for the two ORFs (**Figure 4B**). The raw RNA reads obtained from planktonic *C. albicans* culture aligned against the DNA sequence revealed that while continuous RNA reads could be uniquely mapped spanning the full-length orf19.297, only spurious RNA reads could be mapped against the shorter orf19.279. This indicates that the orf19.279 is either a pseudo-gene or, is not transcribed in planktonic cells. Based on the crystal structure of the homologous DTD enzyme from *E. coli* (PDB id: 1JKE_A, (Ferri-Fioni, Schmitt et al. 2001)), the protein (corresponding to orf19.279) would lack a putative active-site residue **K7** i.e. lysine residue at 9th position in the protein sequence (**Figure 4C**), rendering it non-functional, if expressed at all.

2.2 Loss of DTD1 function affects cell viability *in S. cerevisiae* but not *C. albicans*

The functional significance of the DTD enzyme has been well characterized previously (Soutourina, Blanquet et al. 2000). To validate the result and explore its *in vivo* role in *C. albicans*, strains lacking the functional *DTD* gene were created. The *S. cerevisiae* laboratory strain BY4741 (Winston, Dollard et al. 1995) and *C. albicans* SC5314 strains were used for homozygous deletions (Gillum, Tsay et al. 1984). The *S. cerevisiae dtd1* strains were confirmed by PCR analysis and *C. albicans dtd* strains were confirmed by Southern hybridization (see **Materials and methods**, **Figure 13** and **14**). Contrary to the previously published report, we found that *S. cerevisiae dtd1* mutants exhibit profound growth defect (**Figure 5A**) as well as a significant reduction in cell viability in rich media like YPD. There is a

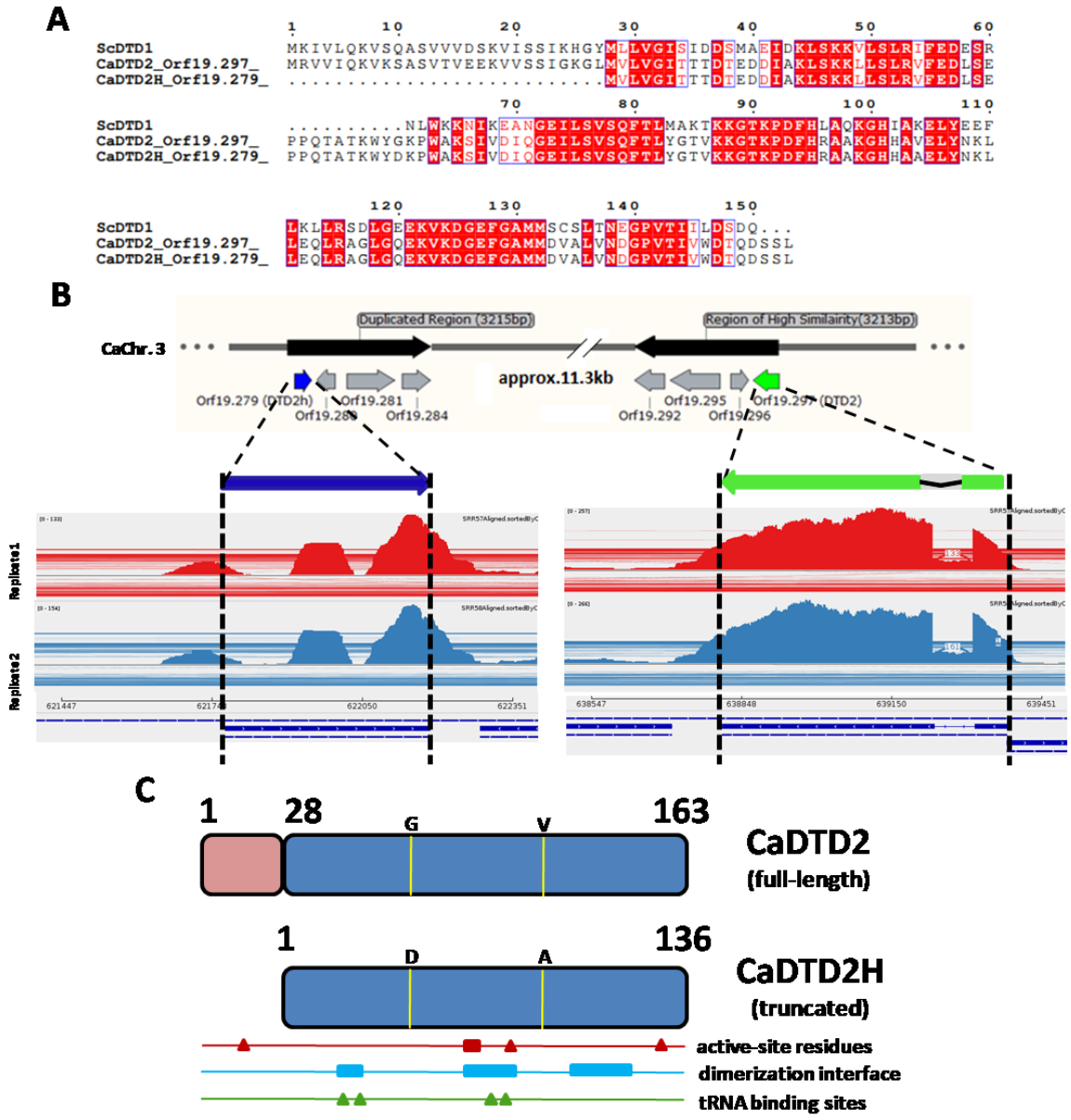


Figure 4. *C. albicans* possesses two *DTD* genes homologous to *S. cerevisiae DTD1*.A. Amino acid sequence alignment of *S. cerevisiae* DTD1 (150 amino acids) and two *C. albicans* putative DTD proteins-CaDTD2 (Orf19.297) and the Orf19.279 product, containing 163 and 136 amino acids, respectively. The ScDTD1 and CaDTD2 show more than 57% sequence identity**. B.** (**Top**) Genetic architecture of *DTD* genes *C. albicans* 3rd chromosome. The two ORF's belong to a ~3.2kb region duplicated region inversely separated by approx.11.3 kb on both the chromosomes. (**Bottom**) The raw RNA reads from planktonically grown *C. albicans* were aligned against the genome. While full length ORF19.297 (green) shows continuous RNA reads, only spurious reads could be obtained against the truncated orf19.279 (blue). **C.** Domain comparison of the putative full length CaDTD2 vs. truncated CaDTD2H, where N-terminal sequence region is absent. Blocks of amino acid residues important for enzymatic activity are indicated. The altered amino acid residues at C-terminal domain (blue) are indicated as yellow bars.

reduction of cell viability from 90% in wild-type to 48% in *dtd1* mutants (**Figure 5B, C**). However, no observable phenotypes were noticed for *C. albicans* wild-type vs. mutant strains (**Figure 5D, E, F**). Thus, despite the absence of exogenously added D-amino acids, DTD function seems to be important for growth and viability.

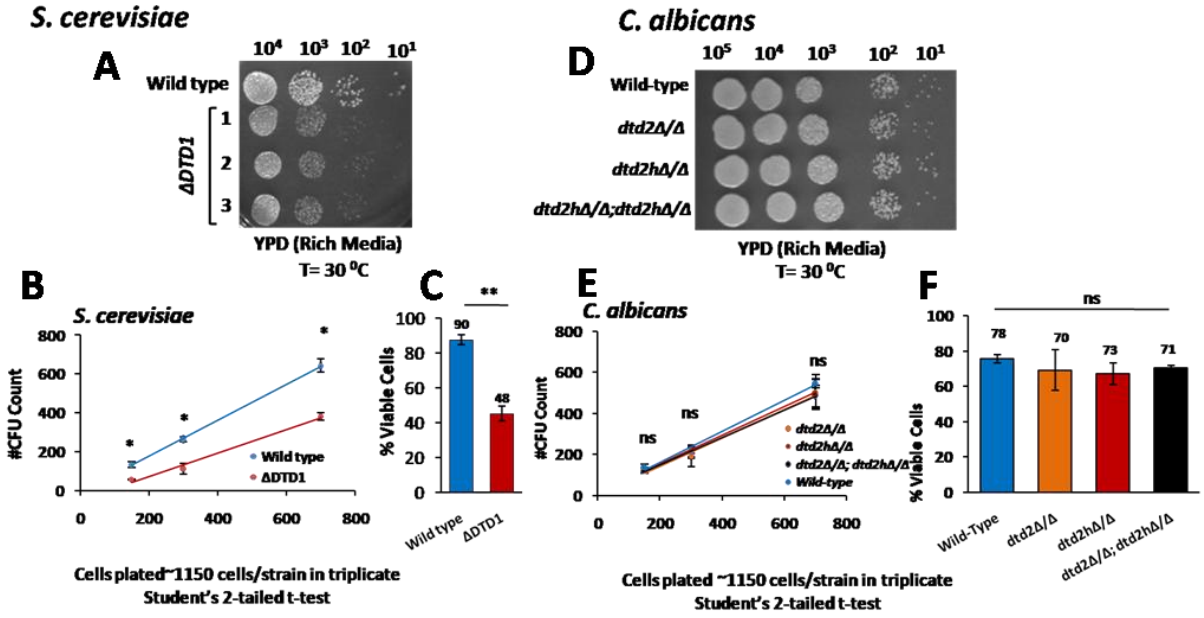


Figure 5. Loss of *DTD* affects cell viability and growth in *S. cerevisiae* in rich media. Cells from overnight YPD cultures of **A.** *S. cerevisiae* wild-type and three independent *dtd1* mutants, and, **D.** *C. albicans* SC5314 derived strains (wild-type, full-length *dtd2*Δ/Δ*, truncated dtd2h*Δ/Δ, and strain lacking all four copies *dtd2*Δ/Δ*; dtd2h*Δ/Δ were spotted on YPD plates. Approximately 150, 300 or 700 cells of *S. cerevisiae*, **B** and *C. albicans*, **E** were plated on YPD plates and colony forming units were counted. **C** and **F** show the quantification of loss of viability of both organisms. Data were pooled from three independent experiments. Statistical significance was determined by Student's 2-tailed t-test (ns- no difference>0.05, * p<0.05, ** p<0.001)

2.3 Unlike *S. cerevisiaedtd1* mutants, *C. albicans* mutant strains do not exhibit toxicity toward D-tyrosine and D-leucine

It is well-understood that the formation of D-aminoacyl-tRNA, *in vivo*, accounts for the toxicity of D-amino acids in *E. coli dtd*, or *S. cerevisiaedtd1* strains which exhibit growth defect at 100 μM D-tyrosine (Soutourina, Blanquet et al. 2000, Soutourina, Soutourina et al. 2004). The D-amino acid toxicity assay was performed on minimal media agar plates with *C. albicans* mutant strains at 0 mM, 2 mM, and 10 mM D-tyrosine. However, unlike *S. cerevisiae* mutants, no effect on growth was observed in *C. albicans* strains (**Figure6**). This indicates that *C. albicans* can tolerate a high level of D-tyrosine even when DTD function is lost. One possibility is that this organism possesses additional level checkpoint to prevent D-amino acids incorporation into protein biosynthesis. A second possibility is that unlike *S. cerevisiae, C. albicans* is able to utilize D-amino acids as a source of nutrients.

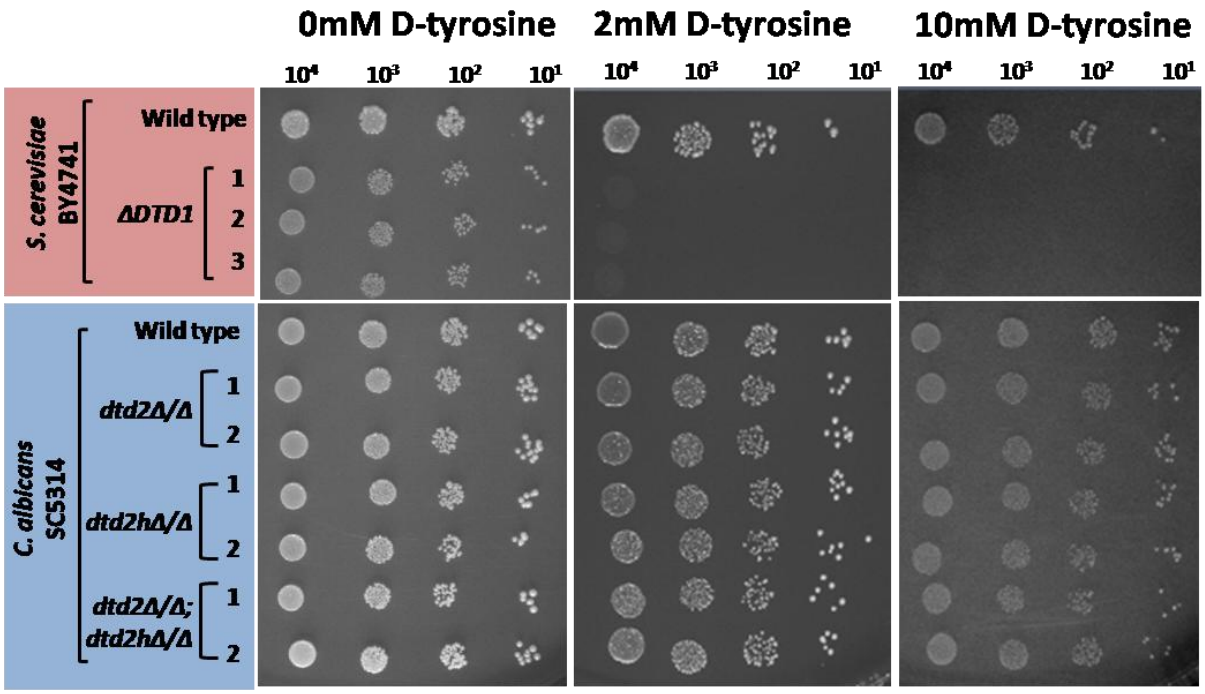


Figure 6. *C. albicans dtd* mutants do not exhibit toxicity to a high level of D-tyrosine. The top panel shows the effect of D-tyrosine on *S. cerevisiae* wild-type and three independent *dtd1* transformants. Mutant cells cease to grow beyond 2mM D-tyrosine. However, no noticeable difference in growth was observed among *C. albicans* strains (wild-type, full-length *dtd2*Δ/Δ, *truncated dtd2h*Δ/Δ, and a strain lacking all four copies *dtd2*Δ/Δ*; dtd2h*Δ/Δ*).*At least two independent transformants of *C. albicans* mutant strains were used for phenotypic comparison.

2.4 *C. albicans* full-length *DTD2* (orf19.297) can complement *S. cerevisiae dtd1* mutant against D-amino acid toxicity

DTD is an evolutionarily well-conserved enzyme displaying a high level of sequence identity (Wydau, van der Rest et al. 2009). Despite these two organisms being phylogenetically separated by millions of years of evolution (Shen, Zhou et al. 2016) where one is free-living and the other obligate residence on the human body, we hypothesized that DTD from one organism may functionally complement the other or vice versa. Since *S. cerevisiaedtd1*mutantsdisplay a phenotype on D-amino acid-containing media, we tested whether *C. albicans DTD* genes can functionally complement *S. cerevisiae* mutant. To test this, *C. albicans* full-length Ca*DTD2* (orf19.297) or truncated Ca*DTD2H* (orf19.279) were PCR amplified and inserted into yeast centromeric vector pRS316 with *URA3* as a selectable marker (**Figure 7A**). The resultant plasmids pRS316-CaDTD2 and pRS316-CaDTD2H along with *S. cerevisiae DTD1* containing plasmid pRS316-ScDTD1 were introduced separately into *S. cerevisiae dtd1* mutant. We observed that the full-length Ca*DTD2* gene containing strain, *i.e. S. cerevisiae* ΔDTD1(pRS316-CaDTD2) shows significant growth rescue in presence of 1 mM D-tyrosine, while the strain transformed with Ca*DTD2H* gene continue to exhibit growth defect in presence of D-tyrosine (**Figure 7B**). This experiment established the full-length *CaDTD2* as a genuine *DTD* gene. However, no functional information regarding the other gene *CaDTD2H* could be ascertained.

2.5 *C. albicans* can utilize D-Leucine as a nutrient source, but not by *S. cerevisiae*

The lack of a noticeable phenotype of *C. albicans dtd* mutants even in the presence of D-amino acid prompted us to ask whether they are utilizing the D-amino acid as a nutrient source. It is known that many bacterial species in the natural environment can utilize D-amino acids as a nitrogen source, and thereby help to recycle the same in the environment (Zhang and Sun 2014). To test this, we generated all the three classes of *dtd* mutants- *dtd2*Δ/Δ*, dtd2h*Δ/Δ and a strain lacking both genes *dtd2*Δ/Δ *dtd2h*Δ/Δ in a *C. albicans* SN148 background which is auxotrophic for three amino acids leucine, arginine, and histidine along with uridine (Noble and Johnson 2005). The *dtd* deletion strains were confirmed by Southern hybridization analysis (**Figure 8A**). D-amino acid toxicity assay was performed on minimal media agar plates containing L-leucine, D-leucine or both. The *S. cerevisiae* as well as *C. albicans* strains, all of which are leucine auxotrophic, exhibit growth in the presence of 500 µM L-leucine (**Figure 8B, panel1**). In the

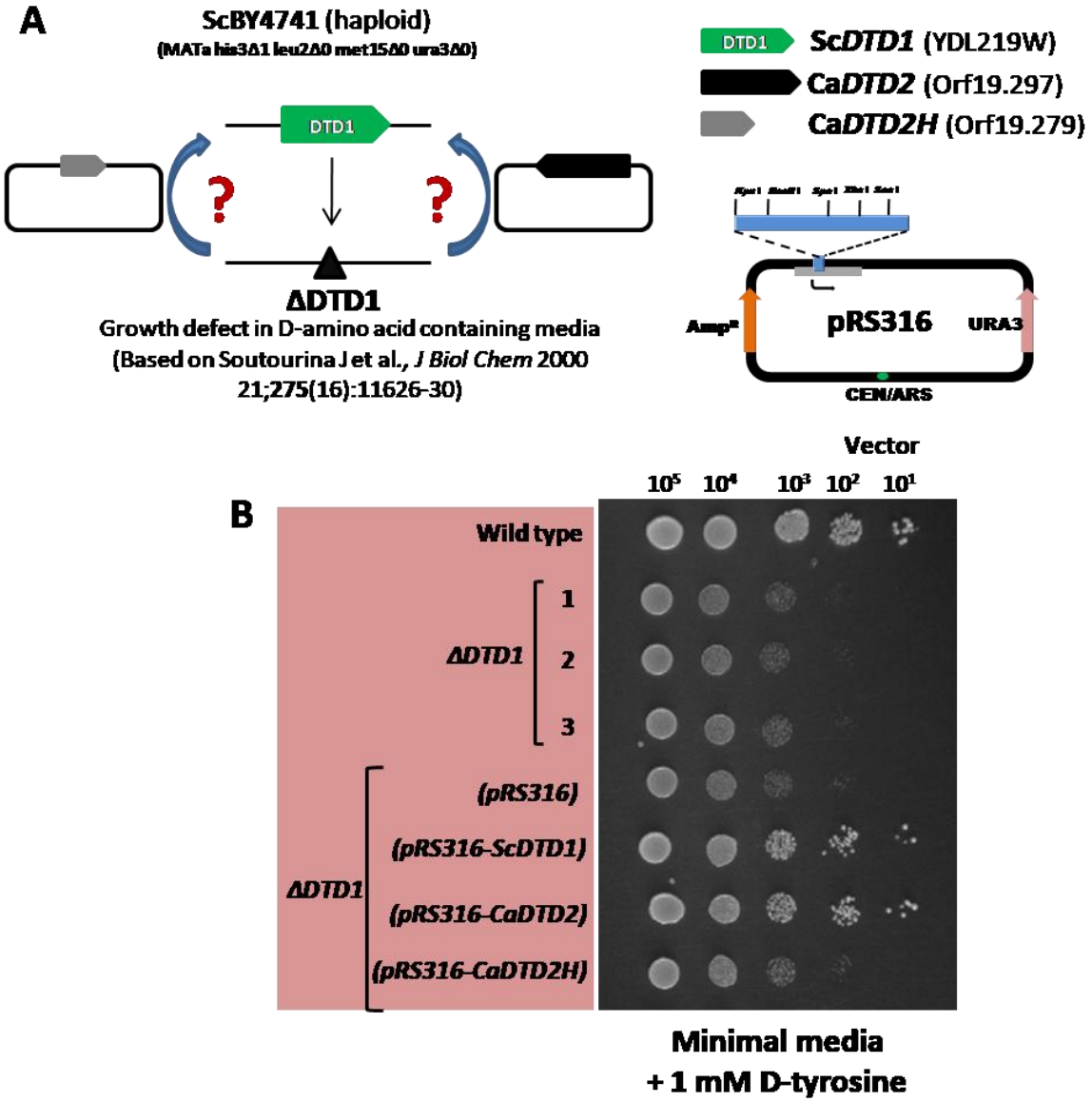


Figure 7. Functional rescue by *C. albicans* gene in *S. cerevisiae dtd1*strain.A. The schematic of functional rescue experiment. **B.** While full length Ca*DTD2* (strain ΔDTD1(pRS316-CaDTD2) could completely rescue the growth phenotype in presence of 1 mM D-tyrosine, the truncated CaDTD2H (strain ΔDTD1(pRS316-CaDTD2H) could not rescue the phenotype. The strain ΔDTD1 (pRS316-ScDTD1) which shows phenotype rescue serves as control for the above strains.

absence of leucine, auxotrophic strains are unable to grow (**Figure 8B, panel2**). In the presence

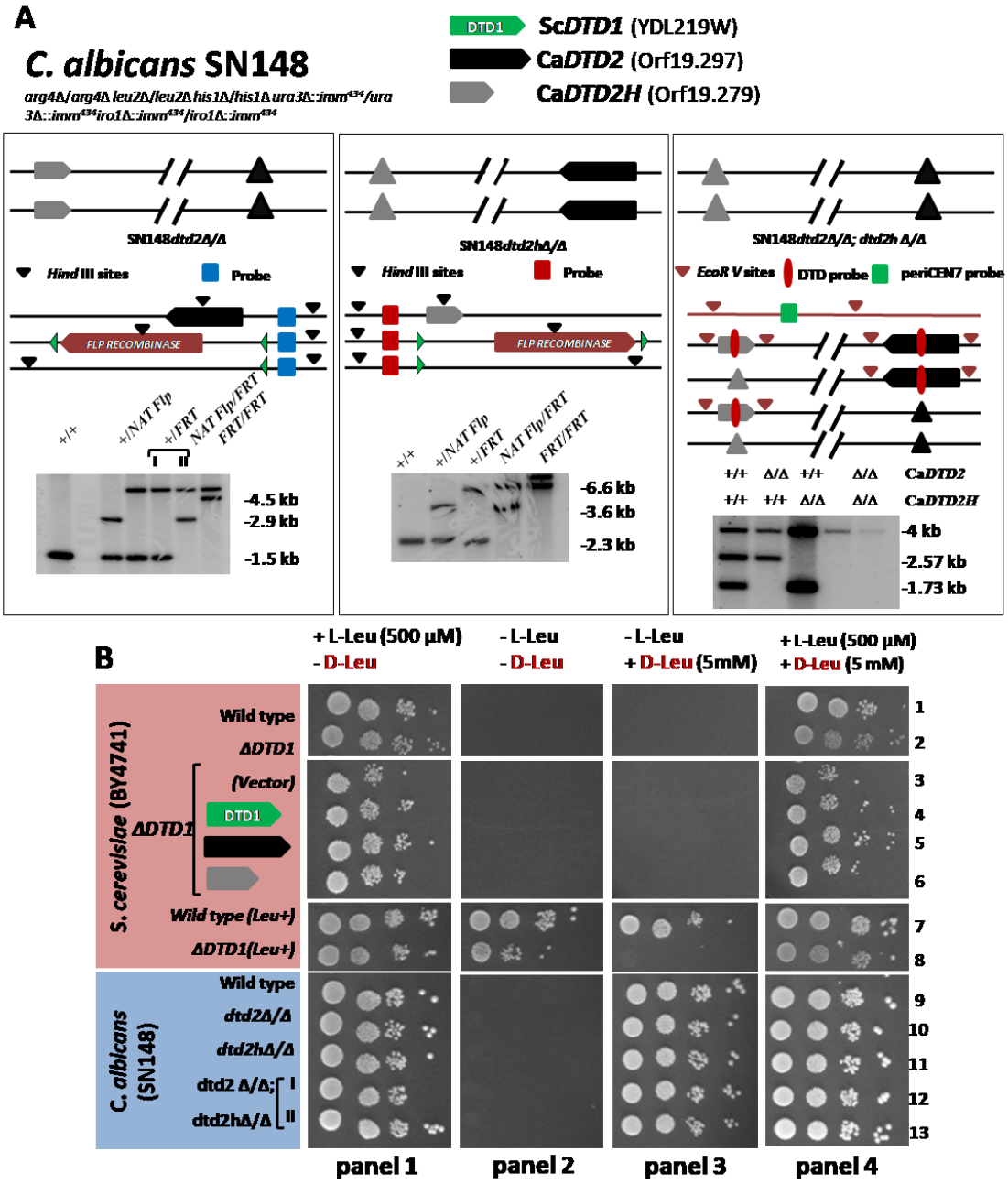


Figure 8. ***C. albicans*** **utilizes D-amino acid as nutrient source. A.** Schematic showing disruption of *DTD* genes in C. albicans SN148 strain background. All deletion strains were confirmed by southern analysis. **B.** D-amino acid toxicity assay on minimal media agar plates. The *S. cerevisiae* as well as *C. albicans* strains, all of which are leucine auxotrophe, exhibit growth in presence of 500 µM L-leucine (panel 1). In absence of leucine, auxotrophic strains are unable to grow (panel 2). However, introducing centromeric vector pRS315 which contain *LEU2* marker rescues growth defect in absence of leucine (see 7,8). In presence of only D-leucine, none of the S. cerevisiae autotrophic strains could grow including ΔDTD1 (Leu+) strain (see 8). However, *C. albicans* wild-type as well as all the *dtd* strains utilizes D-leucine and grow normally (panel 3). Supplementing L-leucine in D-leucine containing media readily rescues growth defect in S. cerevisiae strains (panel 4).

of only D-leucine, none of the *S. cerevisiae* autotrophic strains could grow including *dtd1*(Leu+)

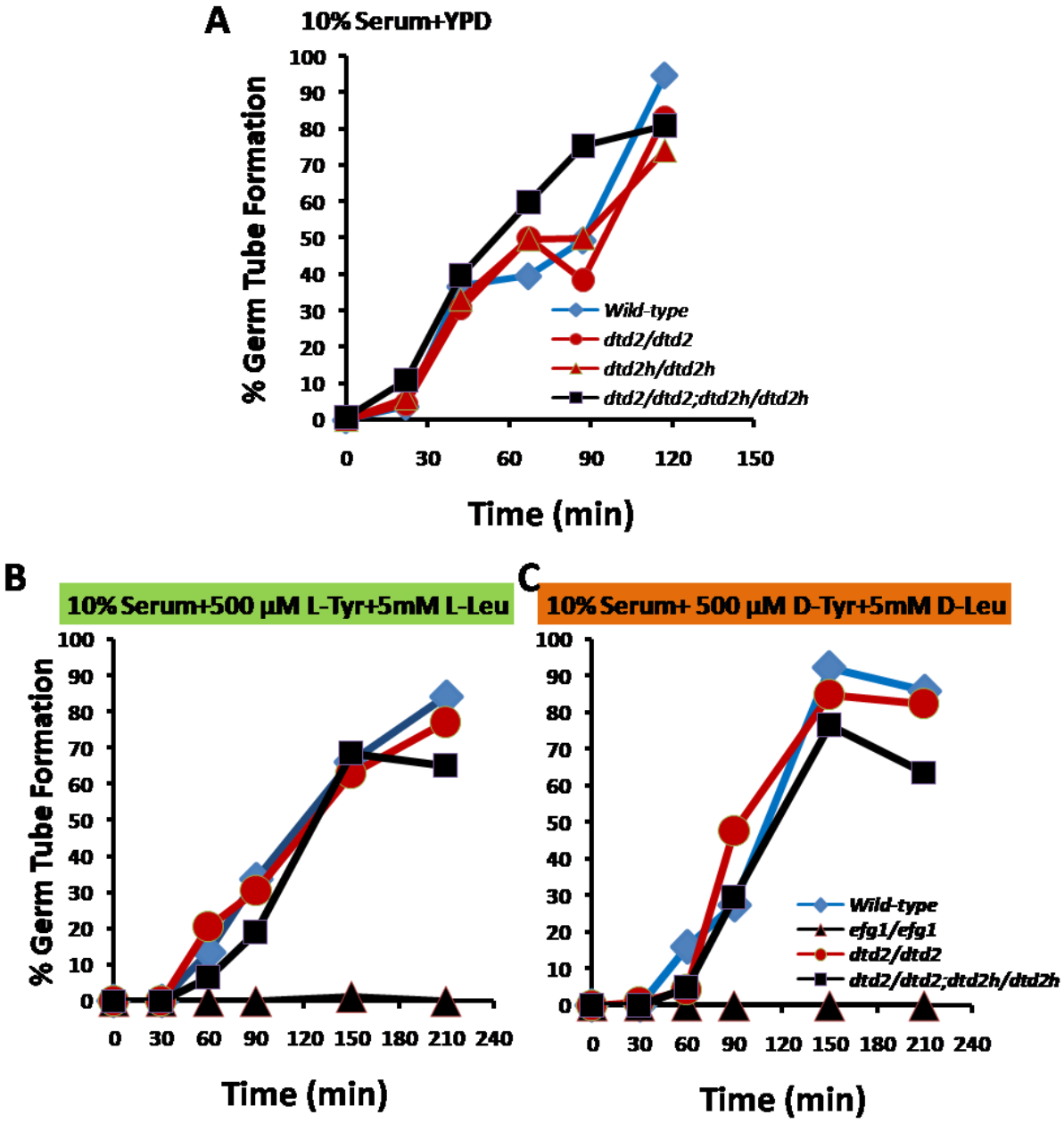


Figure 9. Kinetics of germ tube formation in *C. albicans dtd* mutants. The wild-type parental strain (SC5314, the *dtd2 (full length* null mutant), the *dtd2h* (truncated null mutant) and the strain null for both *dtd2* and *dtd2h* were grown overnight in YPD(+80 µg/mL uridine) at 30 ^{o}C and then 0.02 OD_{600}/ mL culture (absorbance at 600nm) were inoculated into **A.** YPD, **B.** L-amino acid, or **C.** D-amino acid containing media along with 10% serum. The proportion of cells forming germ tubes was determined by bright field microscopy at indicated intervals. The *dtd* null mutants did not have defect in germ tube formation in presence of D-amino acid in the media.

strain (**Figure 8B, panel3**). However, *C. albicans* wild-type as well, as all the *dtd* strains, could

utilize D-leucine and grow normally. This observation has one important implication as to how a pathogenic fungus is able to turn the otherwise toxic D-amino acids into a nutrient source. The free-living *S. cerevisiae* strain could not do that. While this ability is independent of DTD function (since *dtd* strains also utilize D-leucine like wild-type stain), the lack of phenotypic growth defect observed previously in *C. albicans dtd* mutants (**Figure6**) can be explained by this observation. Similar studies with other auxotrophic markers, including histidine and arginine have to be tested to make the observation more conclusive.

2.6 Yeast-to-hyphal transition is unaltered in *C. albicans dtd* strains

C. albicans cells are able to undergo different morphological transitions in response to specific environmental cues (Soll 2002). Some of the well-characterized morphological states include yeast, pseudohyphae, hyphae, opaque, chlamydospores, gastrointestinally induced transition (GUT), and grey phenotype (Slutsky, Staebell et al. 1987, Berman and Sudbery 2002, Miller and Johnson 2002, Pande, Chen et al. 2013, Tao, Du et al. 2014, Rai, Singha et al. 2018). Among these, switch between the yeast and filamentous form (hyphae or pseudohyphae) is closely associated with pathogenicity (Jacobsen, Wilson et al. 2012) and strain locked in either yeast or hyphae form is avirulent in the mice bloodstream infection model (Braun and Johnson 1997, Lo, Kohler et al. 1997). To test whether loss of DTD function effect the yeast-to-hyphal switch, the formation of germ tube kinetics was followed in hyphal inducing condition, *viz.* in presence of 10% fetal bovine serum at elevated temperature, 37 ^{0}C. No change in the kinetics of germ tube formation was observed in either rich YPD medium or defined medium in the presence or absence of D-amino acids (**Figure 9**). This indicates that loss of DTD function doesn't affect the morphological switching between yeast-form and hyphae under conditions tested.

2.7 D-amino acids prevent biofilm growth of *C. albicans dtd* strains

The D-enantiomers of certain amino acids (tyrosine, leucine, methionine, and tryptophan) are known to trigger biofilm disassembly in bacterial biofilms (Kolodkin-Gal, Romero et al. 2010). Like bacteria, *C. albicans* often form a thick multilayered drug-resistant biofilm structure when the cells come in contact with a biotic or abiotic surface. To test whether loss of DTD

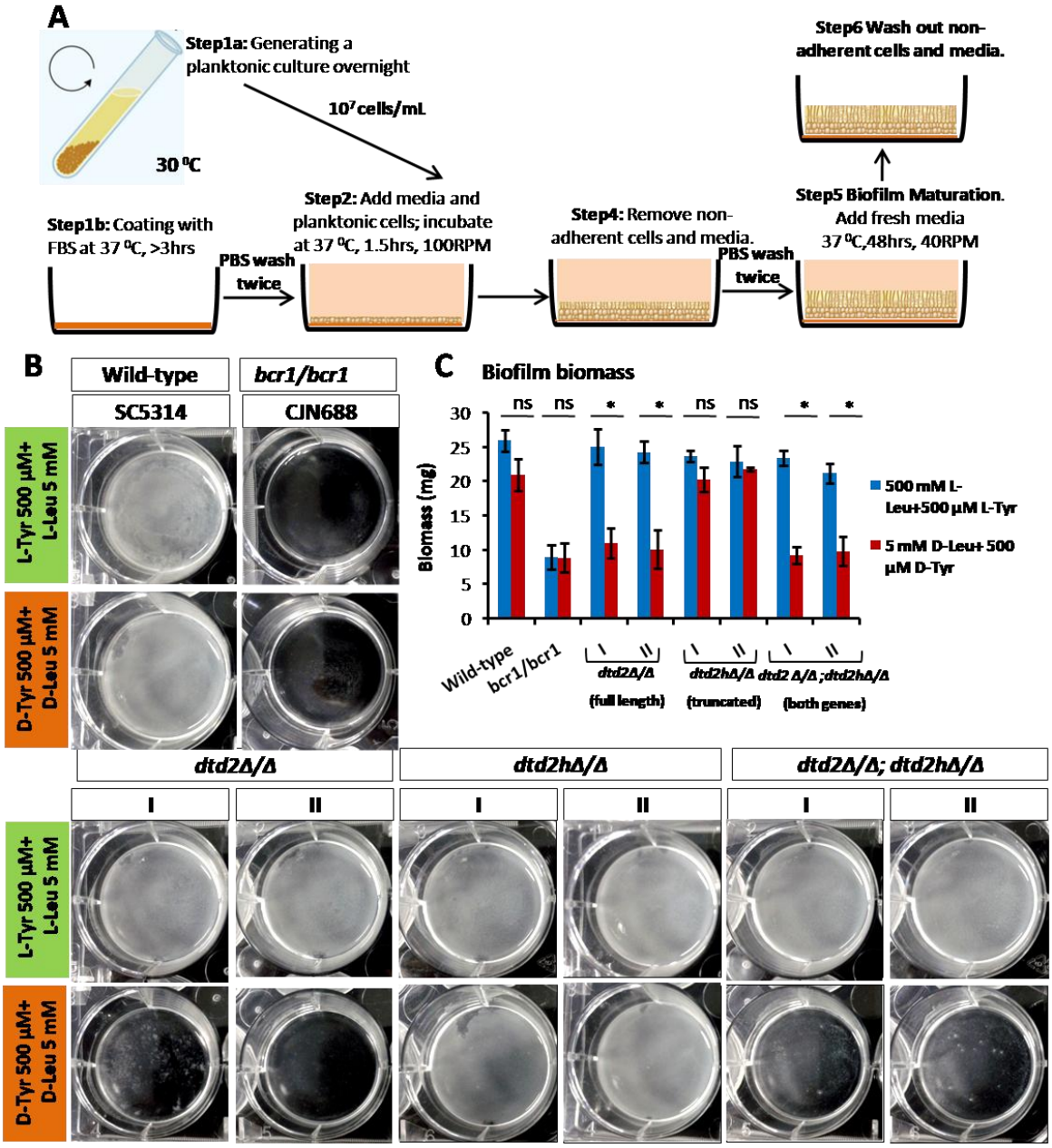


Figure 10. Loss of DTD function prevents biofilm growth in the presence of D-amino acids. A. A step wise illustration of *C. albicans in vitro* biofilm growth assay. **B.** The wild-type parental strain (SC5314), a previously characterized biofilm-defective CJN688 strain, two independent transformants each of *dtd2*Δ/Δ *(full length* null mutant), the *dtd2h*Δ/Δ (truncated null mutant) and the double homozygous deletion strain *dtd2*Δ/Δ *dtd2h*Δ/Δ were assayed for biofilm formation. Presence of 5mM D-leucine+500µM D-tyrosine prevented biofilm growth in strains homozygous for full length DTD2 gene. No affect was seen in strains lacking the truncated DTD2H variant. C. The average total biomass was calculated from three independent samples of each strain. (n=3, error bar: standard deviation; Student's 2-tailed paired *t* test, * $p<0.05$).

function affects the biofilm formation, the biofilm formation on the surface of serum-treated

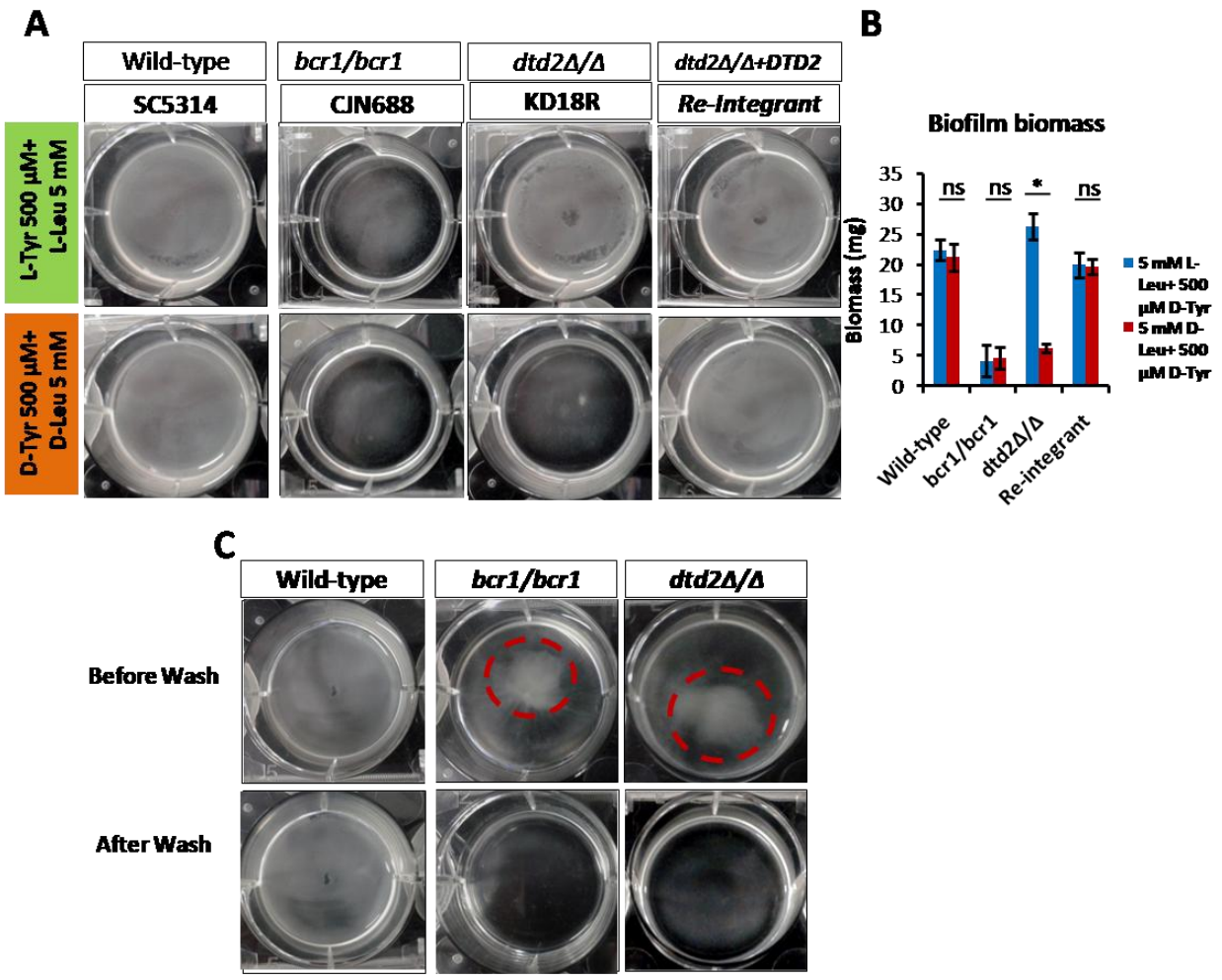


Figure 11. The nature of biofilm defect in *C. albicans dtd* strains .A. The biofilm defect in presence of D-amino acids could be readily rescued by ectopic integration of a functional copy of the*DTD2* gene at RPS1/RPS10 locus. **B.** The average total biomass was calculated from three independent samples of wild-type, *dtd2*Δ/Δ and the reintegrant strain. (n=3, error bar: standard deviation; Student's 2-tailed paired *t* test, *p<0.05). C. The *dtd2* strain fails to adhere in presence of D-amino acids, similar to *bcr1*Δ/Δ. Clumps of planktonically growing cells could be detected (encircled red) before wash, which are easily removed upon washing with 1x PBS (pH 7.0).

polystyrene plates was followed under well-established biofilm-inducing media (YNB-galactose

media) (Chandra, Kuhn et al. 2001) either containing L- or D-form of leucine and tyrosine together. The biofilm assay was based on visual inspection and biofilm dry weight biomass(**Figure 10A**). The Presence of 5mM D-leucine+500µM D-tyrosine in the media prevented biofilm growth in strains homozygous for full-length *DTD2* gene (**Figure 10B, C**). The biofilm defect could be readily rescued by the ectopic integration of one copy of the *DTD2*gene at RPS1/RP10 locus **(Figure 11A)**. No effect was seen in strains only lacking the truncated *DTD2H* variant (**Figure 10B**).

The formation of *C. albicans* biofilm is a multistep process (Lohse, Gulati et al. 2018). During the adherence step, yeast-form cells attach to biotic or abiotic surface. During this proliferation stage, a basal layer forms which consist of yeast, pseudohyphal and hyphal cells. This layer matures into a complex, structured biofilm, in which cells are encapsulated in the extracellular matrix. Strains that are adherence defective, like *bcr1*Δ/Δ fail to form a thick mature biofilm (Nobile and Mitchell 2005) (**Figure 10B, 11B, C**). However, this adherence defective strain can grow planktonically on the plates **(Figure 10C)**. We observe a similar phenotype for *dtd2*Δ/Δ strain (**Figure 11C**) indicating that loss of DTD function probably prevents biofilm formation at adherence step. This observation is preliminary and has to be quantified and tested with standard adherence assays.

3. Discussion

This study provides insights into the relevance of an evolutionarily well-conserved enzyme *in vivo* in two yeast species- *S. cerevisiae*, a free-living harmless organism and, *C. albicans*, an obligate opportunistic pathogen. At the molecular level, DTD is known to enforce homochirality in protein biosynthesis by preventing D-amino acids from entering translational machinery (Soutourina, Soutourina et al. 2004, Ahmad, Routh et al. 2013). It also decouples achiral glycine molecules mischarged on $tRNA^{Ala}$ by alanyl-tRNA synthetase, a role crucial to prevent glycine mistranslation in proteins (Pawar, Suma et al. 2017). In this study, we have focused on the 'chiral proofreading' role. The conserved and indispensable role of this enzyme can be appreciated due to the fact that the *C. albicansDTD2* can rescue the D-amino acid toxicity in an *S. cerevisiae dtd* mutant, despite being phylogenetically separated for millions of years. Secondly, this study unveiled an important aspect of D-amino acid utilization as a nutrient source by *C. albicans*. The *S. cerevisiae* strains which are auxotrophic for leucine fail to grow in the absence of L-leucine. Supplementing D-leucine could not rescue the growth even in *DTD1* wild-type strain indicating that *S. cerevisiae* cannot use it as a nutrient source. Surprisingly, in the absence of L-leucine, we observed that *C. albicans* leucine auxotrophic strains could grow normally when D-leucine was provided instead of L-leucine. Thus, *C. albicans* is able to utilize D-leucine as the nutrient source. Third, we found that *C. albicans* strain lacking a functional *DTD* gene shows no obvious phenotype even up to 10mM D-tyrosine (in contrast, S. cerevisiae *dtd1* mutants showed growth defects at 100 µM D-tyrosine (Soutourina, Blanquet et al. 2000)). Fourth, loss of DTD function did not alter the phenotypic transition between yeast and hyphal from in presence or absence of D-amino acids. However, the presence of D-amino acids renders these strains biofilm defective, an implication important due to its relevance to multi-drug resistance problems. These strains in the presence of D-amino acids phenocopy a *bcr1*Δ/Δ mutant, which can switch between yeast and hyphal or pseudohyphal forms but cannot form a mature biofilm. Whether the adherence defect we observed for *dtd2*Δ/Δ mutants is manifested at the RNA expression level of protein level, further studies have to be carried out.

The fitness of a parasitic organism depends on its ability to extract maximum resources when the competition for its niche is at the upper limit. *C. albicans*, usually being a harmless commensal organism , successfully compete with diverse microbiota in the human host(Berman and Sudbery 2002). Interestingly, bacteria release a lot of secondary metabolites and D-amino

acids constitute one of them (Lam, Oh et al. 2009). The trigger for biofilm disassembly of many bacterial species by multiple D-amino acids gives an indication that it might be a general strategy to release the cells from aging biofilm communities. The same might be true for fungal biofilms but needs further studies to explore this possibility. Our results show that *C. albicans* might be able to thrive in D-amino acid rich conditions by utilizing them as the nutrient source. The limit of poisoning of this fungal pathogen by D-amino acids needs to be further explored. Our results show that despite being able to grow as planktonic cells, they become biofilm defective when DTD function is lost. This observation draws its relevance given that *C. albicans* cells form biofilms on surfaces such as tissues and implanted medical devices and are a source of recurrent infections in immune-compromised patients. The relation between defense mechanisms against D-amino acids, their utilization as nutrient source and role in commensalism and virulence, if any, can be relevant research options that need to be further explored.

4. Materials and Methods

4.1 Strains, plasmids and primers

List of *C. albicans,s* and *S. cerevisiae* strains used in this study are provided in Table 1 and 2, respectively. List of plasmids and oligonucleotide primers used in this study can be found in Table 3 and 4, respectively.

4.2 Media and growth conditions

S. cerevisiae strains were routinely grown in YPD medium(1% yeast extract, 2% peptone, 2% dextrose) at 30 ^{0}C. For growing *C. albicans* strains, additionally, 50 μg/mL uridine was added to the YPD medium. For complementation and /or D-amino acid toxicity assays on solid agar plates, minimal media (2% glucose, 0.17% Yeast Nitrogen Base (YNB) without amino acids or ammonium sulfate, auxotrophic supplements), and 2% agar was used. For biofilm formation, YNB+500mM galactose +amino acid supplements (either D- or L-form as indicated) were used. Germ tube formation kinetics of *C. albicans* strains were measured in YPD and defined L- or D-amino acid-containing media similar to the ones used for biofilm formation were used for the assays.

For cloning, *E. coli* DH5-α (Taylor, Walker et al. 1993)was routinely used and grown in Luria-Bertani) LB media. For selection, LB media were supplemented with 50 μg/mL ampicillin or 34 μg/mL chloramphenicol, whenever required.

4.3 Lithium acetate transformation of *S. cerevisiae* or *C. albicans*

Both *S. cerevisiae,* as well as *C. albicans cells,* were transformed by the standard lithium acetate method as described previously (Ito, Fukuda et al. 1983, Walther and Wendland 2003) with certain modifications adopted to transform either organism. For *C. albicans,* cells from 5mL overnight YPD cultures grown at 30 ^{0}C at 150 rpm were diluted to an optical density at 600 nm (OD_{600}) of 0.3 and grown for additional 4-5 hours until culture reaches an OD_{600} corresponding to 0.8-1.0. Cells were spun down, washed once with 25 mL water, and then with 15 mL freshly prepared LiAc solution (100 mM lithium acetate, 10 mM Tris-HCl, 1 mM EDTA). The pellet was resuspended in 3 times -volume equivalent LiAc solution. To the 100 μL of resuspended cells, 100 μg single-stranded salmon sperm DNA and ~1 μg of appropriate cassette DNA sample was added. To this transformation mix, 600 μL of PEG/LiAc-sol (42% polyethylene glycol 3350

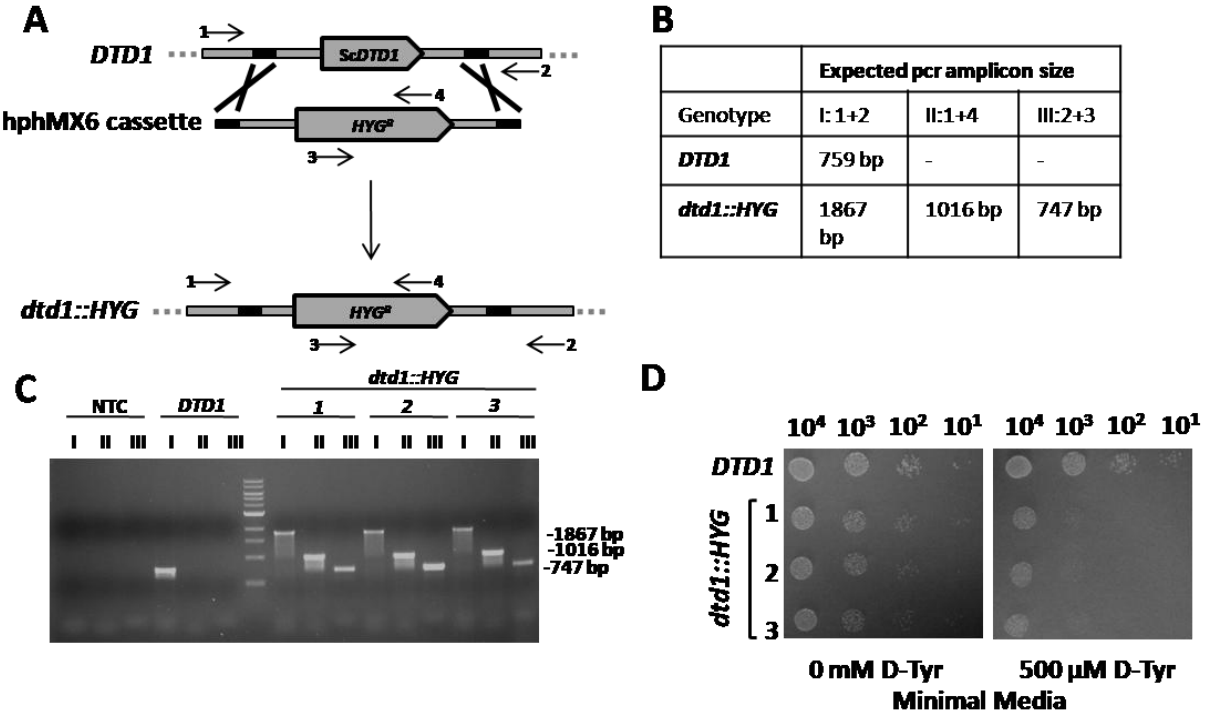
in LiAc-sol) was added and briefly vortexed. The transformation mixture was incubated at 30^{0}C for 12-14 h. A heat shock was given at 44 ^{0}C for 15 minutes. Cells were spun, revived in 1mL YPD for 1-2 hours and plated on selection plates. Plates were incubated for 2-3days at 30 ^{0}C.

For *S. cerevisiae* transformation, to an aliquot of 100 µL of resuspended cells, the following components were added in order: 240 µL of 50 % PEG (w/v), 36 µL of 1M lithium acetate, 100 µg of single-stranded salmon sperm DNA, ~500 ng of the DNA to be transformed, and water up to 360 µL volume was added and briefly vortexed.

Subsequently, the transformation mixture was incubated at 30 ^{0}C for 40 min, followed by heat shock at 42 ^{0}C for 45 min. The cells were revived in 1mL YPD for 1-2 hours and plated on selection plates. Plates were incubated for 2-3days at 30 ^{0}C.

4.4 Deletion of the *DTD1* gene in *S. cerevisiae*

The gene YDL219W in *S. cerevisiae* (Sc*DTD1*) which codes for D-aminoacyl-tRNA deacylase has been functionally characterized previously (Soutourina, Blanquet et al. 2000). Null mutants of *DTD1* in *S. cerevisiae* were constructed in the strain BY4741(Winston, Dollard et al. 1995).. Deletion of the *DTD1* gene was performed by the PCR-based method (Kaster, Burgett et al. 1984) using the hphMX6 cassette as a selectable marker amplified using the plasmid pAG32 (Goldstein and McCusker 1999)(**Figure 11 A**). This cassette contains the aminoglycoside phosphotransferase from *E. coli* fused to the promoter and terminator sequences of the *Ashbya gossypii TEF* gene. Transformants were selected for hygromycin resistance by plating them on YPD with 200 µg/mL hygromycin B. The DNA fragment containing hphMX6 fragment was amplified using the primer pair KD9ScDTD1FP/KD10ScDTD1RP which have 40 base pairs overhands from the upstream and downstream sequences of the *DTD1* gene. Approximately 500 ng of the PCR fragment was used for the transformation of *S. cerevisiae* BY4741 using the lithium acetate method. The deletion of the *DTD1* gene in hygromycin-resistant transformants was verified by PCR amplification of genomic DNA (**Figure 11 B, C**). The phenotype of three independent transformants was confirmed in the presence of D-tyrosine (Soutourina, Blanquet et al. 2000) and used for further experiments.



	Expected pcr amplicon size		
Genotype	I: 1+2	II:1+4	III:2+3
DTD1	759 bp	-	-
dtd1::HYG	1867 bp	1016 bp	747 bp

Figure 12. *DTD1* gene disruption in *S. cerevisiae*. A. Schematic showing deletionof*DTD1* gene by hphMx6 cassette by homologous recombination. **B.** Expected PCR amplicon size for confirmation of transformants. **C.**PCR genotyping showing the amplicons as expected, NTC-no template control, *DTD1*-wildtype, and 1, 2, 3 denote three independent Δ*dtd1* transformants. Roman I, II, III denotes the PCR strategy as shown in **A** and **B**. **D.** Spot dilution assay for the wildtype and three independent Δ*dtd1* transformants on minimal media agar plates in the absence or presence of D-tyrosine.

4.5 Deletion of the putative *C. albicans* DTD genes (Orf19.297 - Ca*DTD2* and Orf19.279-Ca*DTD2H*)

C. albicans is diploid and therefore, it requires disruption of both the alleles of a gene to create a null mutant. To efficiently target either of the *DTD* loci or both, the *SAT1* flipper cassette was used (Reuss, Vik et al. 2004). The cassette contains *C. albicans* adapted nourseothricin resistance marker gene (*caSAT1*) from *Streptomyces noursei* fused to the *C. albicansACT1* promoter and *URA3* transcription terminator sequences. In addition, the cassette possesses the *FLP recombinase* under an inducible maltose promoter (*MAL2p*) and two FRT (**F**LP **R**ecombination **T**arget sequence) sites flanking the *caSAT1* gene. Approximately 500bp

upstream and 500 bp downstream sequences flanking the target locus was amplified by PCR and fused to either side of the FRT sites to create the deletion cassette as explained below.

The DNA sequence downstream (DS) to the *DTD* locus of both the ORF's (CaChr3 coordinates 638053-638719 and 622258-622925) was amplified using primer pair KG424_DTDDSFPSacII/KG425_DTDDSRPSaci and cloned in pSFS2a (Reuss, Vik et al. 2004)as a *Sac*II/*Sac*I fragment to obtain pKD1. The upstream (US) sequences unique to either Orf19.297 (CaChr3 coordinates 639239-639765 amplified using primer pair KD1_DTD_USFPKpnI/KG423_DTD2USRPXhoI) or Orf19.279 (CaChr3 coordinates 621146-621757 amplified using primer pair KG422_DTD_USFPKpnI/KG423_DTD2USRPXhoI), were then independently cloned in pKD1 as *Kpn*I/*Xho*I fragments to generate pKD2 and pKD3, respectively. The clones were confirmed using restriction digestion. Further, pKD2 or pKD3 were digested by *Kpn*I and *Sac*I to release the desired deletion cassette for gene deletion. Approximately 1 μg of the deletion cassette DNA was used for the transformation of *C. albicans* using the lithium acetate method. The deletion of the *DTD* loci (either or both) was confirmed by Southern hybridization (**Figure 11A, B, and C**). At least two independent transformants of each genotype were used for subsequent experiments.

4.6 Cloning of the Sc*DTD1*, Ca*DTD2* or Ca*DTD2H* genes for functional rescue in *S. cerevisiae*

The DNA fragment containing the ORFs Sc*DTD1*, Ca*DTD2* or Ca*DTD2H* (+ at least 1 kb 5' upstream and 3' downstream sequences) were PCR amplified from genomic DNA of *C. albicans* or *S. cerevisiae* using the primer pairs KD11/KD12, KD4/KG425, and KD7/KG425, respectively The PCR products were digested and cloned in pRS316 (Sikorski and Hieter 1989) between the following restriction enzyme sites : a) *Spe*I and *Xba*I for *ScDTD1*, b) *EcoR*I and *Sac*I for *CaDTD2*, and c) *Kpn*I and *Xba*I for *CaDTD2H*to generate the plasmids pRS316-ScDTD1, pRS316- CaDTD2 and pRS316-CaDTD2H, respectively. In the absence of a restriction site, prior to insertion into the pRS316 vector, artificial restriction sites were created by ligating the PCR fragments with pTZ57R_T (Thermoscientific, #K1214) to generate a new restriction site. The clones were confirmed by restriction analysis prior to transformation into the *S. cerevisiae dtd1* strain BY4741ΔDTD1. These were assayed for complementation using D-amino acid toxicity.

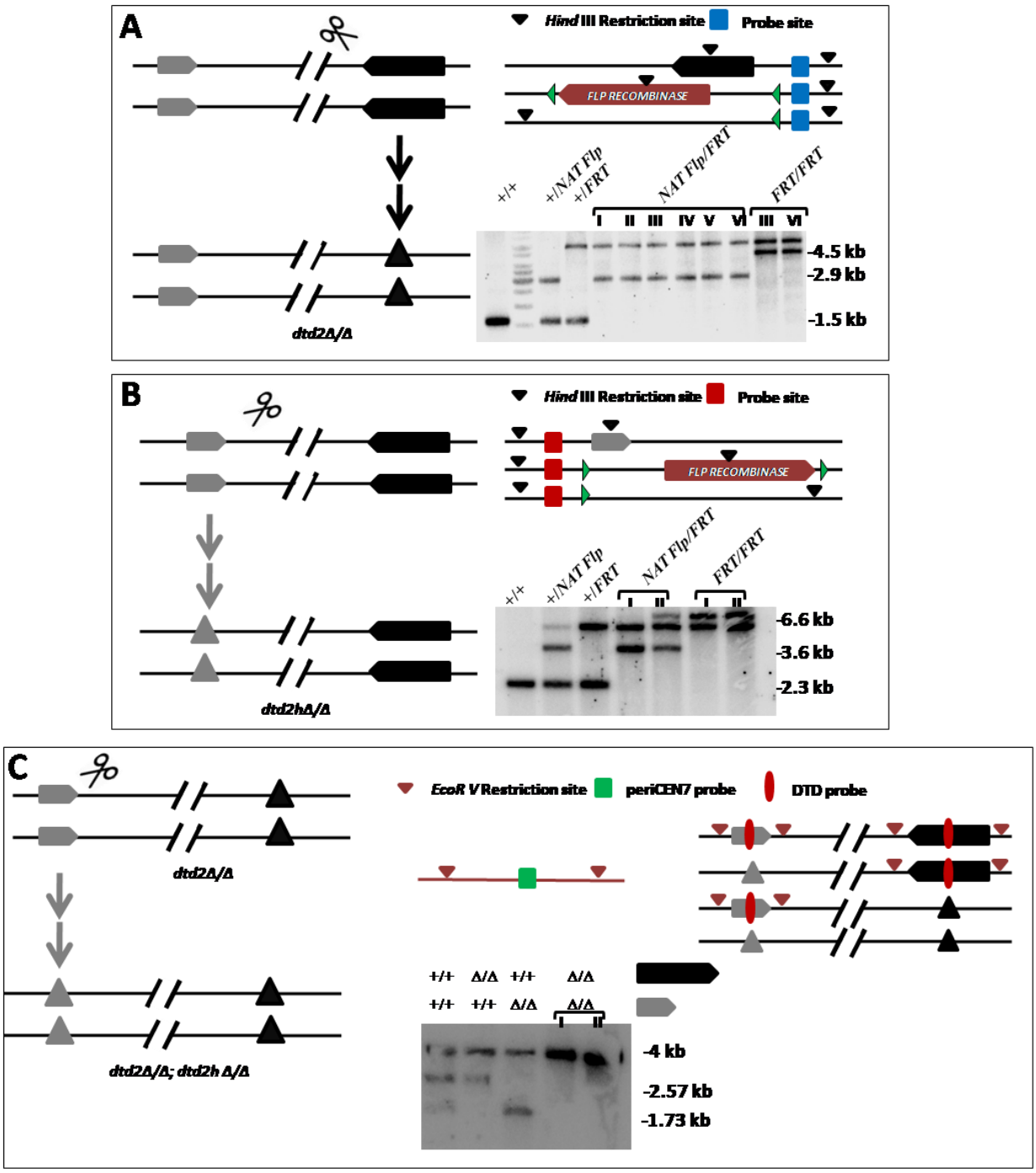


Figure 13*DTD* gene deletion in *C. albicans*. The left panel in each figure shows the schematic of Southern hybridization analysis. Grey box denotes the truncated Orf19.279 (*CaDTD2H)* and the black box denotes full-length Orf19.297 (*CaDTD2*) gene. The black triangles indicate the replacement of the target locus with *SAT1*cassette, either recycled or un-recycled. The *Hind*III-digested genomic DNAs from the strains were blotted and hybridized with probes specific to either Ca*DTD2*, **A**, or Ca*DTD2H*, **B**, or both, **C** (using *Eco*RV restriction sites). Upon replacement of the target locus with recyclable SAT1 cassette, unique restriction patterns for either gene emerge. These restriction patterns further change upon recycling of the cassettes. The periCEN7 probe (**C**) serves as an internal control for *Eco*RV digested genomic DNAs, which remain unchanged in all the strains.

4.7 D-amino acid toxicity assay

Toxicity assays were performed in minimal media in the presence or absence of D-amino acid(s) as indicated. An exponentially grown culture of approximately 2.0 x 10^7 cells per ml density was harvested and washed in 1x PBS (pH 7.0). It was then washed in 0.5% potassium acetate solution and resuspended in 2mL of 1% potassium acetate (+auxotrophic supplements). The cell suspension was incubated at 30 ^{0}C for 48h at 150 rpm. The cells were serially diluted (10 times) and spotted on minimal agar plates containing 0.17% YNB without amino acids and ammonium sulfate, 2% glucose, 50 μg/mL of auxotrophic supplements, and D-amino acid(s) as indicated. The plates were incubated at 30 ^{0}C for 1-3 days and photographed.

4.8 Cell viability Assay

Cell viability assays were performed in YPD media. The desired strains of *S. cerevisiae* (BY4741 and BY4741ΔDTD1) and *C. albicans* (SC5314, KD15R, KD18R, 9T4R5, 9T17R7, T8RT4R and T25RT3R) were grown overnight in 5 mL YPD medium at 30 ^{0}C at 150 rpm. The cells were counted using a hemocytometer and appropriate dilutions were made in 1x PBS (pH 7.0). Approximately150, 300 and 700 cells of each strain were placed on YPD plates in triplicates. The plates were incubated at 30 ^{0}C for 2 days and colony forming units (CFU) were counted. The percentage of cell viability was counted using the following equation:

$$\text{Percentage of cell viability (\%)} = \frac{\text{CFU count on the YPD agar plate}}{\text{Number of cells plated}} \text{x } 100$$

4.9 Germ-tube formation kinetics

C. albicans strains were grown overnight in the yeast form in 5mL YPD at 30 ^{0}C at 150 rpm. Cells were washed with 1x PBS twice and a 0.02 OD_{600}/mL (absorbance measured at 600nm equivalents cells were inoculated to 10mL of the following pre-warmed media: a) YPD+10% fetal bovine serum, and b) YNB+500mM D-galactose+10% fetal bovine serum+ appropriate concentration of D- or L-amino acid(s). The proportion of cells forming germ tubes was determined under a light microscope at various time points thereafter for each strain using the following equation:

$$\text{Percentage of Hyphal cells} = \frac{\text{Numbers of cells with visible germ tube}}{\text{Total cell count}} \text{x}100$$

4.10 *In vitro* biofilm growth and biomass estimation

In vitro biofilm assays were performed on 6-well or 12-well polystyrene plates. The wells were treated with 1mL of fetal bovine serum for at least 3 h prior to the experiment. Before seeding the cells, the serum was removed from each well and the wells were washed twice with 1x PBS. The strains were revived from glycerol stocks by streaking on YPD. A single colony from the YPD plate was inoculated to 5mL YPD and grown overnight at 30 ^{0}C. The cells were spun down, washed twice with 1x PBS. The dilution of cells was adjusted to 0.5 OD_{600} equivalent cells/ mL and seeded on the wells. The total volume was adjusted with media-(YNB 500mM D-galactose with L- or D-amino acid(s)). The plates were incubated at 37 ^{0}C at 100 rpm for 1.5h. Residual media and non-adherent cells were removed washed twice with 1x PBS and fresh media were added to each well. The plates were then incubated at 37 ^{0}C with gentle agitation (40 rpm) for 2 days for mature biofilm formation. Following incubation, the spent media and non-adherent cells were removed, the plate was washed twice with 1x PBS and biofilms were photographed.

For biomass estimation, cells from the mature biofilm were scrapped using a sterile spatula, resuspended in ~200 μL 1x PBS and allowed to dry at 65 ^{0}C. The average biomass for each strain was calculated from three independent samples.

4.11 Construction of *C. albicans* reconstituted strain expressing functional *DTD2* gene

To confirm the *DTD* loss of function phenotype observed in presence of D-amino acid, *RPS1*/*RPS10* integration cassettes were constructed for generating reconstituted strains in *C. albicans*. A DNA fragment containing the *CaDTD2* (Orf19.297, CaChr3 coordinates 638053-640630) was amplified from strain SC5314 using the primers KD4 and KG425 and cloned into pTZ57R_Tto generate two *Sac*I sites on either side flanking the cassette. The cassette released by *Sac*I digestion was cloned into the corresponding site of pBS-SAT1-RP10 plasmid (previously constructed in the lab) which has caSAT1 as a selectable marker and *RPS10* locus cloned for single-site integration. The resulting plasmid pBS-SAT1-RPS10-CaDTD2 was linearized using *Stu*I, and transformed in *dtd2*Δ/Δstrain KD018R giving rise to the reintegrant*dtd2*Δ/Δ*; RPS10/rsp10:: DTD2* (strain KD018RReInt.43). Three independent transformants were validated using Southern blot analysis (**Figure 13**).

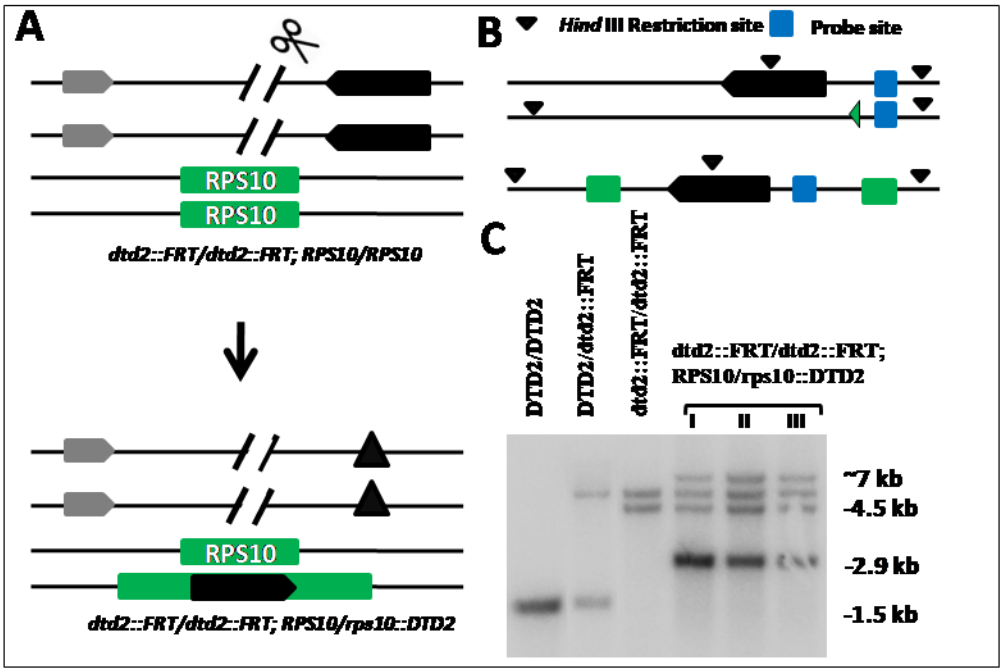


Figure 14 Reintegration of a functional CaDTD2 at an ectopic *RP10* locus. **A**. Schematic showing the ectopic integration at *RPS10* locus. **B.** Line diagram showing the Southern blot strategy for *CaDTD2* reintegration at ectopic *RPS10* locus. The *Hind*III digested genomic DNA was probed with a unique probe site (blue box). The inverted black triangles indicate *Hind*III cut sites. **C**. Southern blot showing *CaDTD2* integration at RPS10 locus-three independent transformants (I,II,III) were probed along with the controls-wild type (*DTD2/DTD2*), single copy (*DTD2/dtd2::FRT*) and the homozygous mutant (*dtd2::FRT/dtd2::FRT*).

4.12 Southern Hybridization

All strains were confirmed using standard Southern hybridization technique as described previously(Southern 1975). Genomic DNA was extracted from the strains to be tested by growing them in 10mL YPD culture overnight at 30 ^{o}C (+100 µg/mL nourseothricin, for NAT^{R} strains) by glass beads method. Approximately 10 µg of RNase A treated genomic DNA was then digested with 20U of the appropriate restriction enzyme for at least 3h. The digested DNA was transferred to a Zeta-probe membrane (BioRad 162-0153)using capillary transfer. The blots were hybridized overnight with the radiolabeled probe (T**able 3**) at 65 ^{0}C, washed, dried and exposed on a phosphor screen for 4 h. The images were captured using Typhoon FLA700 scanner (GE Healthcare).

4.13 RNA sequence analysis

The raw RNA sequence data were downloaded from SRA database (GEO accession no. GSE99902) ((Azadmanesh, Gowen et al. 2017) and mapped on *Candida albicans* genome using STAR software (Dobin, Davis et al. 2013). The expression of the two DTD loci – orf19.297 and orf19.279 was visualized using Integrative Genomics Viewer (IGV) (Robinson, Thorvaldsdottir et al. 2011).

Table 1*C. albicans* stains used in this study

Strain	Genotype	Strain Background	Reference
SC5314	*Wild-type clinical isolate*		(Gillum, Tsay et al. 1984)
SN148	*Δura3::imm434/Δura3::imm434 Δhis1::hisG/Δhis1::hisG, Δarg4::hisG/Δarg4::hisG,Δleu2::hisG/Δleu2::hisG*		(Noble and Johnson 2005)
KD001	*dtd2::NAT Flp/DTD2*	SC5314	This study
KD002	SN148 *dtd2::NAT Flp/DTD2*	SN148	
KD003	*dtd2h::NAT Flp/DTD2H*	SC5314	
KD004	SN148 *dtd2h::NAT Flp/DTD2H*	SN148	
KD005	*dtd2::FRT/DTD2*	KD001	
KD006			
KD007	SN148 *dtd2::FRT/DTD2*	KD002	
KD008			
KD009	*dtd2h::FRT/DTD2H*	KD003	
KD010			
KD011	SN148 *dtd2h::FRT/DTD2H*	KD004	
KD012			
KD013	*dtd2::FRT/dtd2::NAT Flp*	KD005	
KD014			
KD015			
KD016		KD006	
KD017			
KD018			
KD015R	*dtd2::FRT/dtd2::FRT*	KD015	
KD018R		KD018	
KD09T4	*dtd2h::FRT/dtd2h::NAT Flp*	KD009	
KD09T4R5	*dtd2h::FRT/dtd2h::FRT*	KD09T4	
KD09T17	*dtd2h::FRT/dtd2h::NAT Flp*	KD009	
KD09T17R7	*dtd2h::FRT/dtd2h::FRT*	KD09T17	
KD07T2	SN148 *dtd2::FRT/dtd2::NAT Flp*	KD007	
KD07T2R3	SN148 *dtd2::FRT/dtd2::FRT*	KD07T2	
KD011T21	SN148 *dtd2h::FRT/dtd2h::NAT Flp*	KD011	
KD011T21R1	SN148 *dtd2h::FRT/dtd2h::FRT*	KD011T21	

KD0T8	*dtd2::FRT/dtd2::FRT;dtd2h::NAT Flp/DTD2H*	KD015R	
KD0T8R	*dtd2::FRT/dtd2::FRT;dtd2h::FRT/DTD2H*	KD0T8	
KD0T25	*dtd2::FRT/dtd2::FRT;dtd2h::NAT Flp/DTD2H*	KD018R	
KD0T25R	*dtd2::FRT/dtd2::FRT;dtd2h::FRT/DTD2H*	KD0T25	
KD0T34	SN148 *dtd2::FRT/dtd2::FRT;dtd2h::NAT Flp/DTD2H*	KD07T2R3	
KD0T34R	SN148 *dtd2::FRT/dtd2::FRT;dtd2h::FRT/DTDH*	KD0T34	
KD0U4R	SN148 *dtd2::FRT/dtd2::FRT;dtd2h::FRT/dtd2h::URA3*	KD0T34R	
KD0U21R		KD0T34R	
KD0T8RT4	*dtd2::FRT/dtd2::FRT;dtd2h::NAT Flp/dtd2h::NAT Flp*	KD08TR	
KD0T25RT3		KD0T25R	
KD0T8RT4R	*dtd2::FRT/dtd2::FRT;dtd2h::NAT Flp/dtd2h::FRT*	KD08TRT4	
KD0T25RT3R		KD0T25RT3	
KD018RReInt.43-7	*dtd2::FRT/dtd2::FRT;dtd2h::FRT/dtd2h::FRT; RPS10/rps10::DTD2*	KD018R	
KD018RReInt.43-8			
KD018RReInt.43-9			

Table 2 *S. cerevisiae* strains used in this study

Strain	Genotype	Strain background	Reference
BY4741	MATa*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	-	(Winston, Dollard et al. 1995)
BY4741ΔDTD1	MATa*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 dtd1Δ::HYG*	BY4741	This study
BY4741(pRS315)	MATa*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 dtd1Δ::HYG* (pRS315)	BY4741	
BY4741ΔDTD1(pRS315)	MATa*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 dtd1Δ::HYG* (pRS315)	BY4741ΔDTD1	
BY4741ΔDT	MATa*his3Δ1 leu2Δ0 met15Δ0*	BY4741ΔDTD1	

D1(pRS316)	*ura3Δ0 dtd1*Δ*::HYG* (pRS316*)*		
BY4741ΔDT D1(pRS316-ScDTD1)	MATa*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 dtd1*Δ*::HYG* (pRS316-ScDTD1*)*	BY4741ΔDTD1	
BY4741ΔDT D1(pRS316-CaDTD2)	MATa*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 dtd1*Δ*::HYG* (pRS316-CaDTD2*)*	BY4741ΔDTD1	
BY4741ΔDT D1(pRS316-CaDTD2H)	MATa*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 dtd1*Δ*::HYG* (pRS316-CaDTD2H*)*	BY4741ΔDTD1	

Table 3 List of plasmids used in this study

Plasmid Name	Description	Reference
pSFS2a		(Reuss, Vik et al. 2004)
pKD1	pSFS2a+downstream of Ca*DTD2* or Ca*DTD2H*	This study
pKD2	pKD1+upstream of Ca*DTD2*	
pKD3	pKD1+upstream of Ca*DTD2H*	
pKD4	pSKD3 *casat1::URA3*	
pRS315	Yeast centromeric vector with LEU2 marker	(Sikorski and Hieter 1989)
pRS316	Yeast centromeric vector with LEU2 marker	
pRS316-ScDTD1	pRS316+*ScDTD1*	This study
pRS316-CaDTD2	pRS316+*CaDTD2*	
pRS316-CaDTD2H	pRS316+*CaDTD2H*	
pBS-SAT1-RPS10	*RPS1/RP10* gene cloned into the *Not*I site in pBS-SAT1 plasmid	
pBS-SAT1-RPS10-CaDTD2	Plasmid for ectopic integration of *CaDTD2* at *RPS10*	This study
pTZ57R_T	For blue-white screening selection	Thermo scientific, #K1214
pAG32	hMX6 (hygromycin) cassette for gene disruption in S. cerevisiae	(Goldstein and McCusker 1999)

Table 4 List of primers used in this study

Primer	Sequence (5' to 3')	Description
KD1_DTD2_USFP_KpnI	ATAAGGTACCGCAGACTATGCGTTCCAGTTG	Ca*DTD2* US forward primer
KG422_DTD2USFPKpn1	AGATGGTACCACTTTATCAAGCAAGATGATCGG	Ca*DTD2H* US forward primer
KG423_DTD2DSRPXho1	AGATCTCGAGGTTGCTCAAGGTGTTAGTGG	Ca*DTD2/DTD2H* US reverse primer
KG425_DTD2DSRPSacI	AGTAGAGCTCTGACTAGTTCCTGCTGAACTG	Ca*DTD2/DTD2H* DS reverse primer
KG424_DTDDSFPSacII	AGATCCGCGGCCCAAGTCATAATCGTGACG	Ca*DTD2/DTD2H* DS forward primer
KG420_DTD2_ORF_FP	TATTGCAAAGCTCTCCAAGAAGC	Ca*DTD2/DTD2H* ORF forward primer
KG421_DTD2_ORF_RP	CCATCGTTGACCAAGGCAAC	Ca*DTD2/DTD2H* ORF reverse primer
KD2_DTD2_US_delconf	CACTGTACAATGTATTACCAGCC	Primer annealing US of Ca*DTD2* for deletion confirmation
KG425_DTD2_Dconf	ATAGGCTTCAGTGTCAAGCG	Primer annealing US of Ca*DTD2H* for deletion confirmation
KG80	CAGTGGCTACAACTCAGAGCACGC	NAT promoter (*MAL2p*) forward primer
PJ3	CTATTCTCTAGAAAGTATAGGAACTTC	FRT Reverse Primer
KD3_DTD2_Hyd_FP1	CTTCACCGCATGCCCAATCAC	Primer pair for *CaDTD2*(Orf19.297) Southern probing
KD4_DTD2_Hyb_RP	AACATACCCGCTGCAGGTTAATC	
KD7_DTD2H_SFP	CCGTGACATACGCGTTACCAA	Primer pair for Ca*DTD2H*(Orf19.279) Southern probing
KD8_DTD2H_SRP	TGTGTGTGATATTGGGCGTCAC	

30PeriCEN7del2	GAG CGG CCG CCA TCA CGA TAA ACA TTT GGA TGA TAT AAT G	Primer pair for control probe for Southern analysis
30PeriCEN7del1	CGG AGC TCC AAA GAA ATA CCG TTG AAT CTT TGA CTA CTG C	
KD9ScDTD1FP	GTTAATCAAGGCTGCATGTACATATTAAA TAATCCACATACGGATCCCCGGGTTAATT AAG	Primer pair for *S. cerevisiaeDTD1* gene replacement
KD10ScDTD1RP	TTGCTTTTTATTTTCTTCTCGTATATATCT ATAAGGTCAACGACAGCAGTATAGCGAC CAG	
KD11ScDTD1DelconfUSFP	CCTTCGCGTCTCTTATGACG	Primer pair for *S. cerevisiae DTD1* gene deletion confirmation
KD12ScDTD1DelconfDSRP	AACTTCACTGAGAACATAACATGG	
HR40 HygFor	CTCCAACAATGTCCTGACGGAC	Internal primer pair specific to hphMX6 for *S. cerevisiae DTD1* gene deletion confirmation
HR39-Hyg Rev	CACAGTTTGCCAGTGATACAC	

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