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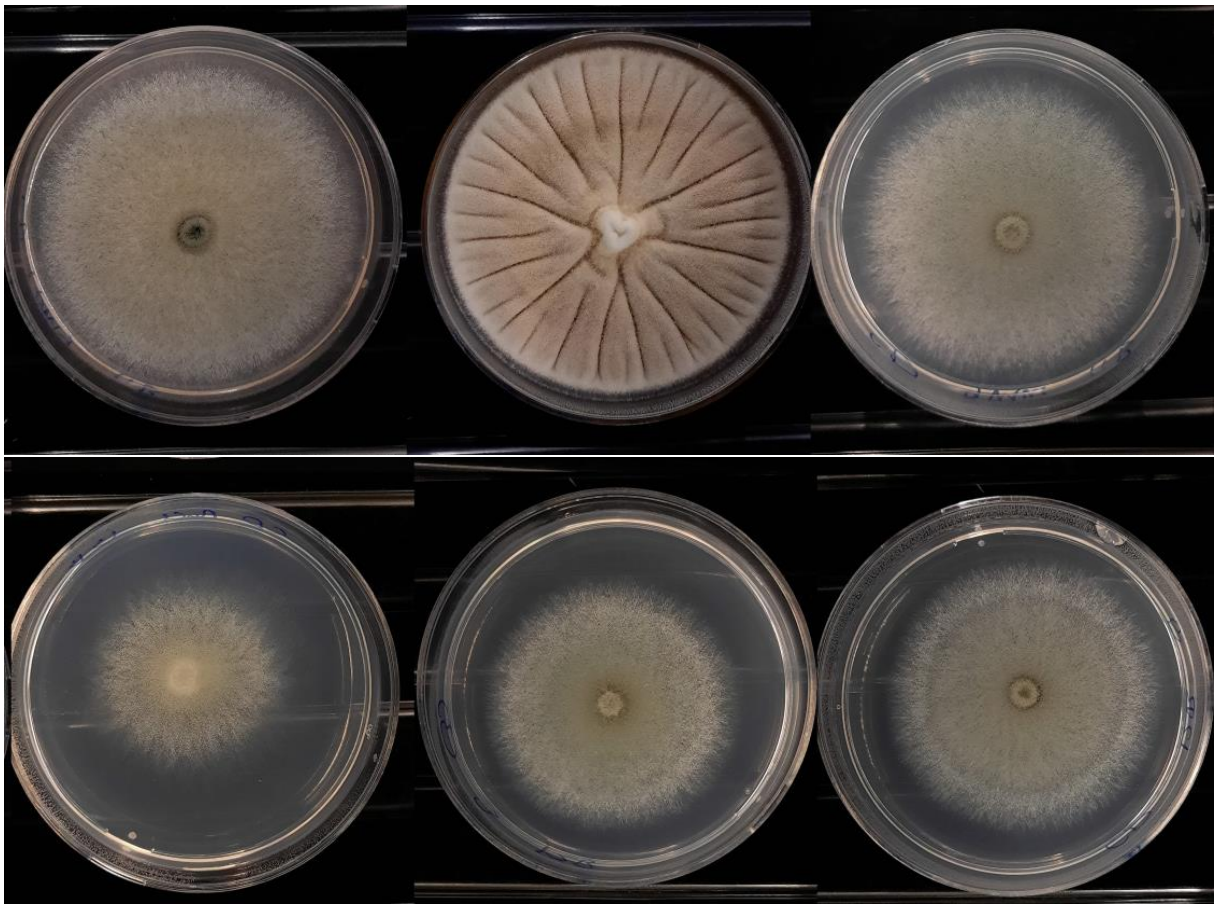

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Utrecht University

Trehalase as novel antifungal target in *Aspergillus fumigatus*

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Samenvatting

Aspergillus fumigatus is een filamenteuze ascomycete schimmel die een saprotrofe levensstijl leidt. Door zijn sporen, die zich door de lucht verplaatsen, kan deze schimmel zich gemakkelijk verspreiden en verschillende omgevingen zoals planten, aarde en dieren koloniseren. Bij mensen kunnen deze sporen in de luchtwegen terechtkomen, waar ze normaalgesproken worden opgeruimd door het immuunsysteem van de gastheer. Dit is echter niet altijd het geval voor patiënten die lijden aan verminderde immunologische afweer ten gevolge van bijvoorbeeld chemotherapie, of longziekten zoals cystische fibrose. Bij deze patiënten kan *Aspergillus fumigatus* zich goed ontwikkelen en veroorzaakt het gezondheidscomplicaties in de longen, zoals de dodelijke manifestatie van aspergillose: invasieve pulmonale aspergillose. Aangezien de beschikbare antischimmelmiddelen voor behandeling en management van aspergillose beperkt is en *Aspergilli* met resistentie tegen antischimmelmiddelen in opkomst zijn, is er dringend behoefte aan nieuwe antischimmelmiddelen. Eerder werd trehalase genoemd als een nieuw molecuul voor antischimmelmiddel-design vanwege de rol bij het ontkiemen van conidiosporen. Daarom richt deze studie zich op meerdere aspecten van trehalase inhibitie, om de potentiële ontwikkeling van een trehalase-blokker te bevorderen. Een van de doelen was om TreB tot expressie te brengen in *E. coli* BL21, op te zuiveren en te gebruiken voor het selecteren van een peptide inhibitor via de RaPID Bioaffinity-assay. Daarnaast werd chemische inhibitie van TreB uitgevoerd met behulp van validamycine A in een EUCAST-gebaseerde protocol, en werd de kiemkinetiek gevolgd en vergeleken met andere (klinisch relevante) *Aspergilli*. Het laatste doel van dit onderzoek was het creëren van een *ΔtreB*-mutant door middel van een protoplast-gemedieerd CRISPR/Cas9-protocol om volledige remming van het enzym te simuleren. Met behulp van het *E. coli* BL21-systeem leek in eerste oogopslag voldoende trehalase te worden aangemaakt. Helaas was de eiwitextractie en -zuivering geen succes. Dit kan te wijten zijn aan het feit dat het eiwit waarschijnlijk niet stabiel genoeg is ten gevolge van een deletie van vier aminozuren die na DNA sequencing werd gevonden. Daarnaast illustreert chemische inhibitie van conidiosporen de hardnekkigheid van de sporen, aangezien het gebrek aan intracellulaire trehalose digestie het kiemingsproces niet lijkt te beperken wanneer andere koolstofbronnen dan trehalose beschikbaar zijn. Ten slotte is er geen succesvolle knock-out stam van *treB* gemaakt. Echter werd aangetoond dat de geprotoplasteerde conidiosporen onverwachte veranderingen in micromorfologie leken te hebben ontwikkeld, gezien dunnere hyphae werden aangetroffen in AMM met trehalose en het ontkiemen van de behandelde sporen minder goed ontwikkelde in AMM met proline. Deze resultaten benadrukken het belang van een voorzichtige benadering van dit soort experimenten, gezien de gebruikte CEA10*Δku80* stam een hogere mutatiefrequentie heeft, waardoor de kans op onverwachte veranderingen in fenotype toeneemt.

Samenvatting (Layman)

Aspergillus fumigatus is een schimmel die vooral plantenresten afbreekt. Omdat de sporen van de schimmel gemakkelijk door de lucht kunnen worden opgepikt, kan deze schimmel zich gemakkelijk verspreiden en in verschillende omgevingen groeien, zoals op planten, dieren of in de grond. Bij mensen kunnen deze sporen in de gezichtsholten en de longen terechtkomen, waar ze meestal worden opgeruimd door het immuunsysteem van de gastheer. Dit is echter niet altijd het geval voor patiënten die al last hebben van andere gezondheidscomplicaties, zoals het ondergaan van chemotherapie, of genetische aanleg en/of longziekten zoals cystische fibrose. Bij deze patiënten ontwikkelt *Aspergillus fumigatus* zich goed en kan het een infectie veroorzaken die, indien onbehandeld, een ernstige bedreiging kan vormen voor het welzijn van de patiënt. Omdat de beschikbare medicijnen voor de behandeling van *Aspergillus* gerelateerde-infecties beperkt zijn, en resistentie tegen deze medicijnen steeds vaker voorkomt bij *Aspergillus fumigatus* en andere *Aspergillus*-soorten, is er een dringende behoefte aan het ontwerpen van nieuwe medicijnen. Eerder werd het neutrale trehalase-enzym genoemd als een nieuw anti-*Aspergillus* doelwit vanwege zijn rol bij het ontkiemen van de sporen en de ontwikkeling van de schimmel. Daarom legt deze studie de focus op meerdere aspecten die richten op het blokkeren van de trehalase om de potentiële ontwikkeling van geneesmiddelen te bevorderen. Een van de doelen was om het neutrale trehalase-enzym van *Aspergillus fumigatus* tot expressie te brengen in een *Escherichia coli* bacterie. Dit enzym zou hierna gezuiverd worden voor een test waarbij het trehalase-enzym wordt blootgesteld aan een reeks peptiden en trehalase inhiberende peptiden worden gevolgd. Bovendien werd chemische remming van het neutrale trehalase uitgevoerd met behulp van validamycine A en de kieming en groei gevolgd om de ontwikkeling van de *Aspergillus fumigatus* in kaart te brengen. Dit werd vervolgens vergeleken met andere (klinisch) relevante *Aspergillus*-soorten. Het laatste doel van dit onderzoek was het creëren van een mutant, die niet in staat is het neutrale trehalase-enzym tot expressie te brengen, door middel van genetische modificatie. Dit werd gedaan om volledige blokkering van het neutrale trehalase-enzym door een hypothetische trehalase-inhibitor te simuleren. Met behulp van de *Escherichia coli* bacterie leek het neutrale trehalase van *Aspergillus fumigatus* in eerste opzicht voldoende te worden geproduceerd. Helaas bleef de extractie en zuivering van dit enzym zonder succes. Dit zou te wijten kunnen zijn aan de lage expressie of stabiliteit van het neutrale trehalase. Ook bleek uit het gebruik van een chemische blokker van neutraal trehalase dat sporen zich nog steeds konden ontwikkelen als andere koolstofvoedingsstoffen dan trehalose in de media aanwezig waren. Interessant genoeg werden *Aspergillus nidulans* en *Aspergillus niger* beïnvloed door de chemische blokker wanneer ze werden gekweekt in media die trehalose bevatten, maar *Aspergillus fumigatus* niet. Hoe dit precies komt, is niet duidelijk. Niettemin benadrukt dit het belang van het creëren van mutanten die niet in staat zijn neutraal trehalase te produceren. Ten slotte is het niet met succes gelukt een knock-out-stam van het neutrale trehalase-gen te creëren. Echter werd aangetoond dat de sporen die aan het genetische modificatieprotocol waren blootgesteld, anders leken te groeien, omdat de dikte van de kiembuisjes of de ontwikkeling hiervan minder bleek te zijn in vergelijking met de onbehandelde sporen, wat een onverwachte verandering in uiterlijk was.

Abstract

Aspergillus fumigatus is a filamentous ascomycetous fungus that leads a saprotrophic lifestyle. Due to its airborne spores, this fungus can spread easily and colonize different environments such as plants, soil, and animals. In humans, these spores can end up in the respiratory track, where it is usually cleared by the host's immune system. However, this is not always the case for patients suffering from either immunological problems, for example due to chemotherapy, or genetic make up and/or pulmonary diseases like cystic fibrosis. In these patients, *Aspergillus fumigatus* thrives and causes health complications in the lungs, such as the deadly manifestation of aspergillosis: invasive pulmonary aspergillosis. As the available antifungals for aspergillosis treatment and management are limited, and antifungal resistant *Aspergilli* are emerging, there is an urgent need for novel antifungals. Previously, trehalase has been coined as a novel antifungal target due to its role in germination of conidiospores. Hence, this study focusses on multiple aspects related to trehalase inhibition to advance potential drug development. One of the goals was to express TreB in *E. coli* BL21 and purify it for selection of a novel peptide inhibitor via the RaPID Bioaffinity assay. In addition, chemical inhibition of TreB was performed using validamycin A in a EUCAST-like protocol, and the germination kinetics were followed and compared to other (clinically relevant) *Aspergilli*. The last goal of this research was to create a *ΔtreB* mutant through a protoplast mediated CRISPR/Cas9 protocol to simulate complete inhibition of the enzyme. Using the *E. coli* BL21 system, trehalase initially seemed to be produced sufficiently. Unfortunately, protein extraction and purification remained unfruitful. This could be due to the low expression or stability of the TreB protein since it lacked 4 amino acids, as was revealed after DNA sequencing. Furthermore, chemical inhibition of conidiospores illustrate the versatility of the spores, as the lack of intracellular trehalose digestion does not seem to limit the germination process when other carbon sources than trehalose are available. Interestingly, *Aspergillus nidulans* and *Aspergillus niger* were affected by validamycin when grown in AMM containing trehalose, but *Aspergillus fumigatus* was not. Although not proven in this study, this might be due to either a different three-dimensional configuration of the trehalose-specific binding site in *A. fumigatus* trehalase, or an alternative system that does rescue the germination of *A. fumigatus*, regardless of validamycin A induced TreB inhibition. This does highlight the importance of creating a *ΔtreB* mutant strain, as these hypothesises could then be tested. Finally, a knock-out strain of the *treB* gene has not been created successfully. However, it was demonstrated that the spores exposed to the CRISPR/Cas9 procedure did inherit change in micromorphology, as hyphae were found to be thinner in AMM containing trehalose and germlings were less developed in AMM containing proline. These results highlight the necessary caution upon these kind of experiments as the used CEA10*Δku80* strain has an overall higher mutation rate, thus increasing the odds of observing finding unexpected changes in phenotype.

Abstract (Layman)

Aspergillus fumigatus is a mould that mostly breaks down plant debris. Since the spores – the fungus' seeds – can be easily picked up by the air, this fungus can spread easily and grow in different environments like on plants, animals or in the soil. In humans, these spores can end up in the facial cavities and lungs, where it is usually cleared by the host's immune system. However, this is not always the case for patients who already suffer from other health complications, like undergoing chemotherapy, or genetic make up and/or pulmonary diseases like cystic fibrosis. In these patients, *Aspergillus fumigatus* thrives and causes infection which, when untreated, could pose a serious threat to the wellbeing of the patient. As the available drugs for treating aspergillus-caused infection are limited, and resistance to these drugs is becoming more common for *Aspergillus fumigatus* and other *Aspergillus* species, there is an urgent need for the design of new drugs. Previously, the neutral trehalase enzyme has been coined as a new anti-*Aspergillus* target due to its role in germination of the spores and development of the mould. Hence, this study focusses on multiple aspects related to blocking the trehalase to advance potential drug development. One of the goals was to express the neutral trehalase enzyme of *Aspergillus fumigatus* in an *Escherichia coli* bacterium. This enzyme would be purified for a test in which the trehalase-enzyme is exposed to an array of peptides and trehalase inhibiting peptides would be followed.

In addition, chemical inhibition of the neutral trehalase was performed using validamycin A and the growth and development was to map the development of the *Aspergillus fumigatus*. In addition, this was compared to other (clinically) relevant *Aspergillus* species. The last goal of this research was to create a mutant, which is unable to express the neutral trehalase enzyme, through a genetic modification with CRISPR/Cas9. This was done to simulate complete blockage of the neutral trehalase enzyme by a hypothetical peptide-based blocker. Using the *Escherichia coli* bacterium, *Aspergillus fumigatus* neutral trehalase initially seemed to be produced sufficiently. Unfortunately, extraction and purification of this enzyme remained unfruitful. This could be due to the low expression or stability of the neutral trehalase. Furthermore, using a chemical blocker of neutral trehalase showed that spores could still develop when carbon-nutrients other than trehalose were present in the media. Notably, *Aspergillus nidulans* and *Aspergillus niger* were affected by the chemical blocker when grown in media containing trehalose, but *Aspergillus fumigatus* was not. It remains unclear why this is the case. Nonetheless, this does highlight the importance of creating mutants unable to produce neutral trehalase. Finally, a knock-out strain of the neutral trehalase gene has not been created successfully. However, it was demonstrated that the spores exposed to the genetic modification protocol did seem to grow differently as the thickness of the germ tubes or its development was found to be less compared to the untreated spores, which was an unexpected change in appearance.

Preface

In this thesis, I evaluate my work regarding genetic and microbiological experiments with *Aspergillus fumigatus*. This did not always go smoothly but it was a very interesting and educative experience, nonetheless. To see a visually simplistic cell like a conidiospore develop in mycelia and conidiophores, becoming a bridge between the microbiological and microbiological realm, is mind-blowing. I hope this piece of work will spark your (the reader) interest for the relatively underrepresented field that is (medical) mycology. I would also like to dedicate this chapter to show my gratitude towards Jacq van Neer and Hans de Cock for guiding me throughout this research project, as well as Margot Koster for being the second reviewer. Finally, I would also like to thank everybody in the microbiology group for making my stay at the Kruytbuilding a fond memory.

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19 September 2023

Schoondijke, The Netherlands

Abbreviations

Abbreviation	Meaning
ABPA	Allergic bronchopulmonary aspergillosis
AMM	*Aspergillus* minimal medium
AMP	Ampicillin
CBB	Coomassie Brilliant Blue
CNPA	Chronic necrotizing pulmonary aspergillosis
CPA	Chronic pulmonary aspergillosis
GMMA	Glucose minimal medium agar
IPA	Invasive pulmonary aspergillosis
IPTG	Isopropyl β-d-1-thiogalactopyranoside
KI	Knock-in
KO	Knock-out
LB	Lysogeny broth
PCR	Polymerase chain reaction
ROS	Reactive oxygen species
SAFS	Severe asthma with fungal sensitization
TM	Transformation medium
TreB	Neutral trehalase
WT	Wild type

Content

1. Introduction

Aspergillus is a saprotrophic, filamentous ascomycetous fungus (Phylum: Ascomycota, Class: Eurotiomycetes, Order: Eurotiales, Family: Trichocomaceae). This genus of mouldy fungi can be found in soil, animals, plants, and marine ecosystems [1]–[4], and has first been described in 1729 by Pier Antonio Micheli [5]. He named the fungus after the aspergillum tool, which closely resembles the shape of the *Aspergillus* conidiophores he most likely observed (Figure 1). This aspergillum tool-like shape is characteristic for all 446 species accepted *Aspergillus species* [6][7], and play a major role in the *Aspergillus* lifecycle as airborne conidiospores and conidiospores are produced from the specialized hyphae. As *Aspergillus* could be found all over the world and are capable to digest a wide variety of organic materials, species belonging to this genus play an essential role in the biosphere [8]. Some of these species, like *Aspergillus nidulans* (*A. nidulans*) and *A. niger*, have even been implemented in the biotechnology sector for large-scale production of compounds, such as citric acid [9].

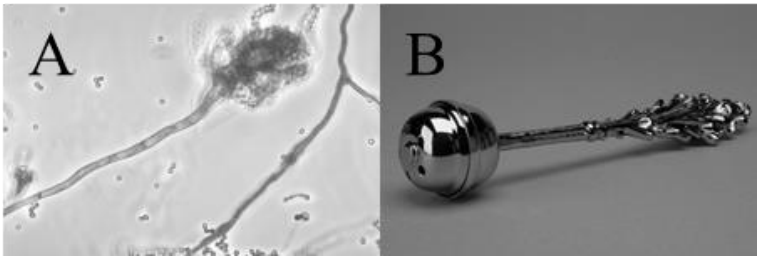

Figure 1: Conidiophore of *Aspergillus fumigatus* (A) and its resemblance to the aspergillum tool (B).

Unfortunately, the *Aspergillus* genus also contains a handful of species that could cause complications in humans and animals alike. Aspergillosis manifests itself in different types of infection, such as sino-nasal infections in dogs and cats [10]. In humans, *Aspergillus* is mainly found as a pathogen infecting the pulmonary system, where aspergillosis could manifest itself in different forms, like non-invasive aspergillomas, allergic bronchopulmonary aspergillosis (ABPA), chronic necrotizing pulmonary aspergillosis (CNPA), chronic pulmonary aspergillosis (CPA), invasive pulmonary aspergillosis (IPA), severe asthma with fungal sensitization (SAFS, Figure 2)[11].

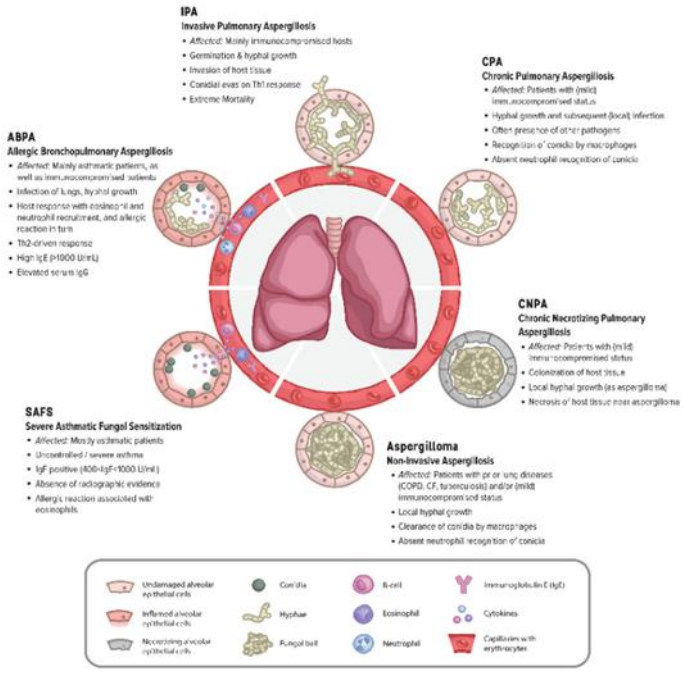

Figure 2: schematic representation of the different aspergillosis diseases [11].

Annually, aspergillosis affects more than 14 million patients, of which around 300,000 cases concern life-threatening IPA with mortality rates reaching 94% [12]. Epidemiological studies have elucidated that *A. fumigatus* is the main contributor to aspergillosis in humans, and the most frequent filamentous fungal pathogen found in tertiary healthcare facilities [13], [14]. This fungus usually infects organ recipients, neutropenic and cystic fibrosis patients, and during severe viral infections [15]. Hence, these patients receive triazole prophylaxis, usually itraconazole, posaconazole or voriconazole [16]. In addition to triazoles, amphotericin B or echinocandins can be deployed when aspergillosis still occurs. However, physicians are becoming more limited in their treatment options due to the rise of antifungal resistance in *A. fumigatus*, such as *cyp51A* mutations or upregulation of the *MDR1* gene [17], [18]. Also, it should be mentioned that extensive antifungal therapy could have severe consequences for the patient's quality of life due to side-effects such as nephrotoxicity. As it is expected that the risk group for Aspergillosis will only increase in the future, while the current complicated treatment strategies become more limited due to the rise of resistance in *A. fumigatus*, there is an urgent need to broaden the arsenal of antifungals. Ideally, novel antifungals target different mechanisms essential to fungal cell growth and integrity to evade cross-resistance. Furthermore, the targeted mechanisms for the novel antifungals must be exclusively present in fungi to minimize unwanted side-effects.

Currently, the accepted antifungals for aspergillosis all target compounds involved in cell wall and – in extend – cell integrity. Although previously believed to be a rather lifeless organelle keeping the cell content together, literature suggests that the fungal cell wall is prone to alteration due to factors deriving from its environment [19], impacting the morphotype [20]. As morphotypes have different cell wall composition, it makes treatment with antifungals targeting cell wall compounds trickier. Hence, it would be interesting to aim for a target that is not directly involved in cell wall maintenance and stability.

The development of a novel antifungal to inhibit conidia germination has recently been coined [11], with the argument that inhibiting fungal growth as early as possible could prevent infection from occurring as the host's immune system would have enough time to clear the conidiospores. Following this approach, disrupting metabolic pathways that occur during the breaking of dormant conidia are interesting targets for designing novel antifungals. Such an example is trehalose, as transcriptomics studies have found that trehalose is involved in the early development of *Aspergillus* conidia [21]. During the dormant state of conidia, trehalose is synthesized to function as a carbon sink, as it could be hydrolysed to glucose through enzymatic activity by neutral trehalase (TreB) [22], [23], a glycoside hydrolase (Figure 3).

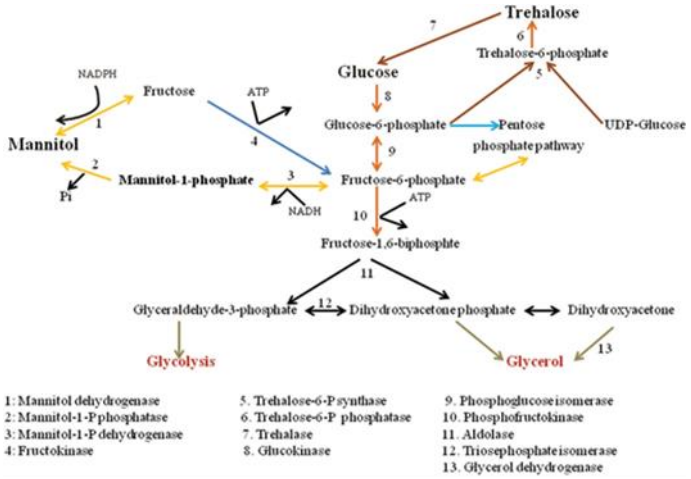

Figure 3: Carbohydrate metabolism and conversion in fungi shows trehalose as a storage molecule of carbon and bottlenecked by enzymatic activity of trehalase [22].

This enzyme is located in the cytosol of the spores and has previously been demonstrated to plays a key role in the integrity of *A. niger* mycelia [21]. Alternatively, a similar pathway is utilized to produce glycerol, which in turn in involved in the swelling of the spore due to an increase of turgor. Finally, trehalose has been found to be a stabilizing factor for oxidative and thermic stress in *A. nidulans*

[24]. This is partially due to the conversion of trehalose into mannitol, a compound that is involved in the neutralization of reactive oxygen species (ROS). Therefore, limiting the mannitol synthesis leads to less tolerance of the pathogenic fungus to its host's immune response. Indeed, literature suggests mannitol biosynthesis and trehalose homeostasis are essential for the survivability of *Aspergilli* [25], [26]. In fact, the correlation of reduced pathogenicity and mannitol production has already been demonstrated through genetic manipulation for *Cryptococcus neoformans* in mice [27] and *Cladosporium fulvum* in tomato plants [28]. Additionally, natural compounds like *streptomyces hygroscopicus* derived validamycin A have proven to be effective trehalase inhibitors. Validamycin A, a polyolic secondary amino compound, hinders the trehalose hydrolysis through competitive binding due to its similar molecular structure (Figure 4). Inhibition of germination by validamycin A has been shown for *A. flavus* and *A. niger*, as well as a synergetic effect of other antifungals such as amphotericin B [29], [30].

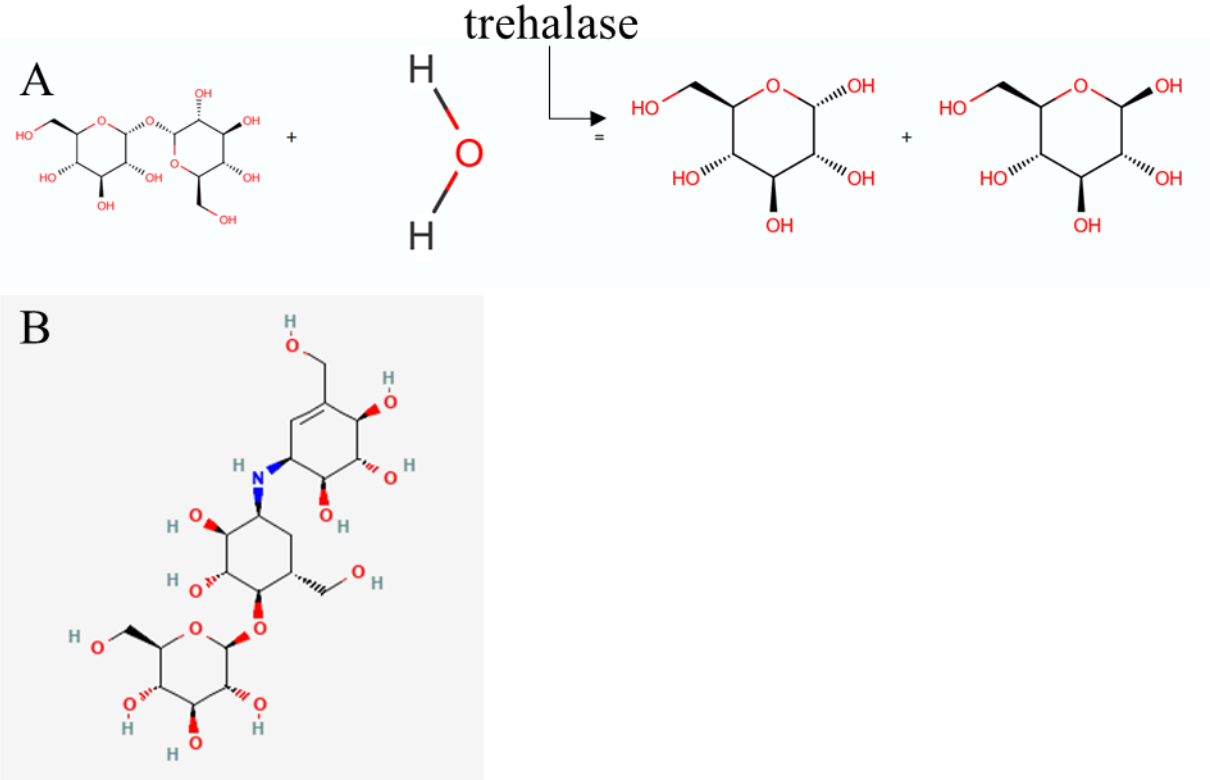

Figure 4: The hydrolysis of trehalose by trehalase results in the release of two glucose molecules (A). The molecular structure of Validamycin A allows for competitive binding to trehalases, blocking the conversion of trehalose into glucose (B).

Considering that trehalose is not metabolized by vertebrates, its abundance in dormant conidia, and its role in cell integrity and germination, inhibiting the trehalase enzyme seems like a safe and promising target for the development of a novel antifungal. Therefore, the aim of this study is to observe the effect of trehalase absence on *A. fumigatus* germination and pathogenicity. To do this, a plasmid-mediated CRISPR-Cas9 knock-out (K.O.) will be performed to simulate the inhibition of trehalase due to a hypothetical compound. The conidial development of the *A. fumigatus ΔtreB* mutant and its stress tolerance will be monitored and compared with non-transformed *A. fumigatus*. In parallel, the *TreB* gene with exclusively the exons will be transformed in BL21 *Escherichia coli* (*E. coli*) to produce and isolate active *A. fumigatus* TreB. Consequentially, the isolated TreB protein could be used in the RaPID bio-affinity assay to select for enzyme inhibiting compounds [31].

2. Materials and methods

2.1. Strains, culture media and growth conditions

The *Aspergillus* species and *A. fumigatus* strains used in this study are described in Table 1. All strains have been revived from a -80°C stock and have been grown on *Aspergillus* minimum medium with 20mM glucose (GMMA) at 37°C for 3 days. Spores were collected and dissolved in 10mL filter-sterilized spore suspension buffer (9% NaCl, 0.005% Tween 20), and stored at 4°C for maximum 2 weeks. Likewise, chemically competent BL21DE3(+) *E. coli* (Lot# SLCP2878, Sigma-Aldrich, St. Louis, U.S.A.) and chemically competent NEB10 *E. coli* were kept at -80°C. Before application, the *E. coli* was first grown overnight on lysogeny broth (LB) agar before use.

Table 1: *Aspergillus* species used in this study.

Species	Strain
A. fumigatus	Af293.1, [32]
	ATCC46645, [33]
	CEA10, [34]
	CEA10*Δku80*, [35]
A. niger	N402, [36]
A. nidulans	FGSC A4, [37]
A. terreus	NHI2624, [37]
A. tubingensis	CBS133792, [38]

2.2. Construction of the knock-in plasmid

To construct the knock-in (KI) plasmid, several steps were be undertaken. This regards extraction of DNA, amplification of *treB*, modification of *treB* through PCR, and ligation.

2.2.1. DNA extraction

A. fumigatus CEA10*Δku80* was inoculated on PDA plates and grown for 3 days at 37°C. The resulting spores were harvested with 1 mL spore suspension buffer (0.85 % (w/v) NaCl and 0.005 % Tween 20). 10 µL fresh spores was used to inoculate 100 ml Transformation Medium (TM, *Aspergillus* minimal medium with 5 g/L yeast extract and 2 g/L casamino acids) in 250 mL Erlenmeyer flasks and incubated for 4 days at 33°C, 200 rpm. Mycelium was harvested using a miracloth filter and washed 3 times with sterile physiological salt solution. Mycelium was added to 2 mL Eppendorf tubes with 2 metal beads (4 mm). The filled tubes were snap frozen with liquid nitrogen and freeze-dried overnight using the FD5512 freeze dryer (IlShinbiobase, Ede, The Netherlands). Subsequentially, the material was crushed using the Qiagen Tissuelyzer (Qiagen, The Netherlands) for 2 minutes at 25 Hz. Fungal DNA extraction was performed using the DNeasy PowerPlant® Pro kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

2.2.2. Amplification of tailed-*TreB*

The amplification of the *TreB* gene carried out by monoplex Touchdown polymerase chain reaction (PCR). Each PCR reaction was performed in a total volume of 25 µL containing 9.75 µL water, 5 µL for both GC-enhancer and reaction mix buffer (New England Biolabs, Leiden, The Netherlands), 2.5 µL dNTP mix (1mM), 1.25 µL 10nM of both forward (5'-tagaggatccccgggTTCGGTTTTAGGGCTCGCACC-3') and reverse primer (5'-aattcgagctcggtacccgggGAGACGCCGGTGGTG-3'), 1 µL of Q5-polymerase (0.5U/µL, New England Biolabs, Leiden, The Netherlands) and 1 µL of 75 ng/µL DNA-template. The amplification was performed by a pre-denaturation step at 95°C for 5 minutes, followed up by 35 cycles. Each cycle has a denaturation step at 95°C for 90 seconds, and elongation at 72°C for 3 minutes. The program started

with 3 cycles containing an annealing step at 79°C and would be repeated with a 9°C lowering of annealing temperature per 3 cycles, until 59.8°C was reached. Finally, a post-elongation step at 72°C was performed for 5 minutes. The amplification products were resolved by 1% agarose gel loaded with ethidium bromide. The target-amplicon was purified using a Nucleospin® gel and PCR clean-up kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and analysed through gel electrophoresis using a 1% agarose gel with ethidium bromide for detection (80 V, 1 hour). Finally, amplicons were stored at -20°C until further application.

2.3. Simulating the inhibition of TreB by a hypothetical compound

As the change in phenotype due to TreB inhibition is still unclear for *A. fumigatus*, experiments like genetic deletion and inhibition through chemical compounds were performed to elucidate potential phenotypical alterations. These experiments are described in the following subparagraphs.

2.3.1. Chemical inhibition of *A. fumigatus* and other *Aspergilli*

To explore the germination dynamics of *A. fumigatus*, the antifungal susceptibility of validamycin A was determined of the *aspergilli* used in this study (see Table 1). This was initially done by adapting the EUCAST protocol for conidia forming moulds in RPMI 1640 and *Aspergillus* minimal medium (AMM) containing 20mM glucose, and data was measured using a synergy H1 (Biotek, Winooski, U.S.A.) [39] at OD 600 using Costar® F-bottom microtiter plates (Corning, New York, U.S.A.) A range of 0.125-128 mg/L validamycin A based on previous findings for *A. flavus* and results were compared to 0 mg/L controls [30]. Hereafter, chemical inhibition of TreB was further explored for *A. niger*, *A. nidulans*, *A. fumigatus* CEA10 and CEA10*Δku80* by repetition in AMM containing trehalose, or proline as carbon source. In addition, data was collected per 30 minutes for 24 hours to map the germination dynamics. These experiments were performed in triplo.

2.3.2. *A. fumigatus* knock-out transformation

A protoplast CRISPR/Cas9 transformation was performed to create *A. fumigatus* CEA10*Δku80ΔtreB* strain according to Peraza *et al.*, with some alterations and is described hereafter [40]. First, spores were inoculated for 20 hours at 30°C, 250 rpm. Next, mycelium was filtered using a double layer of miracloth, and washed with 0.9% NaCl solution. The mycelium was resuspended in 10 mL NMC buffer (0.23M sorbitol, 10mM MES monohydrate, 0.5M $CaCl_2*2H_2O$) containing 60mg homemade lysing enzymes of *Trichoderma harzianum* and incubated for 2 hours at 130 rpm, 30°C. After checking for sufficient protoplasts per view under the microscope, the suspension was filtered over a miracloth in a cut eppendorf. Cold STC (50mM $CaCl_2$, 10mM Tris/HCl pH 7.5, 1.33 sorbitol) buffer was added to a total volume of 45 mL and centrifuged at 2000 rpm for 10 minutes using a swing-out rotor. The spores were resuspended in cold 45 mL STC, and the wash step was repeated. Protoplasts were counted using a Bürker-Türk counting chamber, and 200 µL protoplast suspension (approximately 10^6 protoplasts/mL) was mixed with 10 µg transformation DNA (1µg selection marker, 9 µg rescue template), 50 µL PEG buffer (25% PEG-6000, 50mM $CaCl_2$, 10mM Tris/HCl pH 7.5) and incubated for 20 minutes. This was followed by adding 2 mL PEG buffer, incubation at room temperature for 5 minutes and a subsequent addition of 4 mL STC. Finally, 20 mL minimal medium sucrose top agar (MMST (1x minimal medium, 0.95M sucrose, 0.6% agar, 100 µg/mL hygromycin B) was added to the mix, plated out on MMS plates (1x minimal medium, 0.95M sucrose, 1.2% agar, 100 µg/mL hygromycin B) and incubated for 3 days at 37°C.

Colonies on the selection plates were cut out using a scalp and underwent double purification by culturing on GMMA containing 100 µg/mL hygromycin B. Spores were collected after a final inoculation with GMMA without selection marker.

2.3.3. *A. fumigatus* K.O. screening

To screen for *A. fumigatus* K.O. strains, colony PCR was performed for using colonies on the last purification plate containing the selection marker [41], as well as regular PCR using DNA templates harvested as described above. This was done by collecting spores in 1mL spore suspension buffer and correcting to $1*10^7$ spores/mL. Hereof, 30 µL of spore suspension was exposed to a thermal shock by heating the spore suspensions to 95°C for 15 minutes, followed by cooling down at -80°C for 10 minutes. After thawing, 5 µL of the thawed sample was used as PCR template. For the PCR, 34E forward primer (5'-tcgccattcaggctgGAATTCGGCTCGCACCACTGACTTCTTG-3') and 37E reverse primer (5'-cgcaaaccgcctctcGAATTCGACGATGCTATTGAGAACGAGTCG-3') were used using the same PCR program as mentioned earlier. PCR products were analysed by gel electrophoresis, using a 1% agarose gel (80 V, 1.5 hours).

Simultaneously, phenotypical screening was performed by inoculating $1*10^6$ spores in 200 µL RPMI 1640 or AMM containing trehalose, proline, or glutamate. The germination dynamics followed for 14 strains candidate transformants and a non-protoplasted *A. fumigatus* CEA10*Δku80* spore solution.

The phenotypical screening was extended by introducing spore solutions of the candidates and control to various stresses. This was done for both direct and delayed heat-shock, as described earlier for *A. niger* [42]. To calculate the percentage of heat-resistance per strain, Equation 1 was used. Additionally, $1*10^6$ spores were spotted on RPMI 1640, YPDA (5%) or GMMA plates with or without 2M NaCl, HCl (pH3), NaOH (pH8) or Congo red (25 µg/mL). Finally, these plates were incubated for 3 days at 37°C and fungal growth was compared visually after photographic documentation.

$$\frac{n_{3h,\ 50°C}}{N_{0h,\ 55°C}} = R \qquad (1)$$

Equation 1: ratio of heat-resistance per strain (R), as calculated by the CFU after a delayed heat-shock at 50°C ($n_{3h,\ 50°C}$) over the CFU after a direct heat-shock at 55°C ($N_{0h,\ 55°C}$).

2.4. Obtaining active TreB

To obtain active *A. fumigatus* TreB, *treB* had to be expressed in chemically active BL21DE3(+) *E. coli*. Thereafter, expression of the *TreB* had to be initiated and purification of the protein samples had to be performed. These experiments are evaluated in the following sub-paragraphs.

2.4.1. Transformation of cloned plasmid into BL21DE3(+) *E. coli*

Before initiating the transformation protocol, LB agar plates were prepared with a final ampicillin (AMP) concentration of 50 µg/mL for selection of the transformants. BL21DE3(+) chemically competent *E. coli* was chosen for transformation of an already cloned pET52(+) plasmid with the exons of TreB. The manufacturer's instructions were followed for the transformation and are described hereafter.

Sterile 1.5 mL eppendorf tubes were chilled on ice while BL21 cells were taken from the -80°C freezer and thawed on wet ice for 15 minutes. After complete thawing, 1 µL of the cloned pET52b(+) and 40 µL of cell stock was added to the chilled eppendorf tubes. The DNA/*E. coli* mix was kept on ice for 30 minutes, followed by a 42°C heat shock for 45 seconds. Next, the mix was placed on ice for 2 minutes and 960 µL of room temperature Expression Recovery Medium was added. The culture was incubated for 1 hour at 37°C, 250 rpm. Finally, 200 µL culture was plated on LB agar plates with 50 µg/mL ampicillin and incubated overnight at 37°C.

2.4.2. Protein expression and purification

An overnight culture of transformed BL21 cells was prepared in LB medium, 37°C and 250 rpm. Hereof, 0.5 mL of overnight culture was transferred to a fresh 50 mL LB medium, and incubated for 3 hours at 37°C, 250 rpm. A pET52 plasmid carrying the exons of *treB* (2342 bp, or 86654 Da as protein) was

transformed into chemically competent *E. coli* BL21 cells. The system was initially checked by IPTG-activation of the transformed BL21, followed by boiling for 10 minutes in laemmli buffer. Samples included (un)transformed (non-)activated BL21, and of each 6 µL was loaded on an SDS-PAGE gel (5% stacking gel, 10% running gel). The SDS-PAGE was run at 100 V for 70 minutes and fixed overnight using coomassie brilliant blue (CBB) staining (3mM CBB R-250, 90% methanol and 10 % glacial acetic acid (v/v)). Next, destaining was performed using destain buffer (50% methanol, 40% Milli-Q water and 10% glacial acetic acid (v/v)) until individual bands could be observed by eye.

Hereafter, the expression and purification of active *treB* was performed as described by de Almeida *et al*., with some modifications. Briefly, the culture was activated by the addition of isopropyl β-d-1-thiogalactopyranoside (IPTG) to an end concentration of 100mM and incubated for 16 hours at 20°C, 250 rpm. Consequently, cells were harvested by spinning down by centrifugation for 10 minutes at 8000 *x g* using a 5920 R centrifuge (Eppendorf, Nijmegen, The Netherlands) and resuspended in 1 mL ice-cold lysis buffer (10 mM Tris/HCl, pH 7.0; 100 mM NaCl; 10 mM imidazole; 1 mM phenylmethylsulfonylfluoride and glycerol 10% (v/v)). The suspension was sonified for 3 times 15 seconds at setting 3 (Branson Sonifier model 450) and loaded on a lysis buffer-equilibrated NI-NTA column (Qiagen, Hilden, Germany). This was mixed for 1 hour at 4°C, followed by centrifugation at 300 *x g* and five times washing using wash buffer (20 mM Tris-HCl pH 8.0, 30 mM imidazole, 500 mM NaCl). Elution was performed three times in fractions of 500 µL elution buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl) with an increase of imidazole concentration per elution (50mM, 100mM, and 500mM). Finally, the samples were dialyzed twice using a 30 kDa Ultracell filter (Merck KGaA, Darmstadt, Germany) and 2 x 4 mL dialysis buffer (20 mM Tris-HCl pH 8.0, 200 mM KCl, 10 mM $MgCl_2$). Samples were taken throughout the purification process and compared through SDS-PAGE as described earlier. To test the protein samples for enzymatic activity, a trehalose assay was used (Megazyme, Bray, Ireland) and performed according to the manufacturer's protocol. Finally, to ensure the produced protein was the target protein, amplicons of <1000 bp (see Table *2*) were produced of the *treB* gene in the transformed plasmid. Sanger sequencing was performed at Macrogen (Macrogen, Amsterdam, The Netherlands) and alignment was performed and checked using the multisequence system MAFFT, with an open gap penalty of 1.53, gap extension penalty of 0 and adjusted direction enabled.

Table 2: Primers used to make approximately 1000bp amplicons for sanger sequencing.

Primer	Base pair coverage plasmid	Sequence (5'-3')
1_Ch_Fw	176-1174	GGCGCGCCCTAATAAGCG
1_Ch_Rev		ACCGACTCATTTCACGCATATCC
1_seq_Fw	88-1076	TGGCAGCAGCCTAGGTTAAT
1_seq_Rev		AACCTGAGCTCGACGAATATTTC
2_Ch_Fw	671-1671	CTGGTGCGGTGTCGCAAG
2_Ch_Rev		ACACCGGACTATGTGAGGGATC
2_seq_Fw	497-1503	TATCTCAGTAGGCCCGTCCA
2_seq_Rev		TCTCTCGGCTTGTTGGTCA
3_Ch_Fw	1100-2099	GCCTGGACAAACTCCTTGAAAGT
3_Ch_Rev		CGGACAGGAACATGCAGATAACG
3_seq_Fw	1018-2001	TCTCGAGACGGTAGCTGGTA
3_seq_Rev		GGAACGTACATGCTCTCCAATCT
4_Ch_Fw	1544-2517	CCTCCTGGGACAACAAACGG
4_Ch_Rev		AAGCTTATGCACCGGCGG
4_seq_Fw	1689-2627	GCACATCAAGTCGCAACTCG
4_seq_Rev		GAAGGAGATATACCATGGCAAGC

3. Results

3.1. Production of the pUC19 K.I. plasmid for *A. fumigatus*

To produce a pUC19 K.I. plasmid as rescue template for complementation of a *ΔtreB* mutant, genomic DNA was successfully isolated, with a concentration of 445.2 ng/µL. Amplicons were made with the PCR as described in paragraph 2.2.2 of the Materials and methods, and evaluated using a 1% agarose gel.

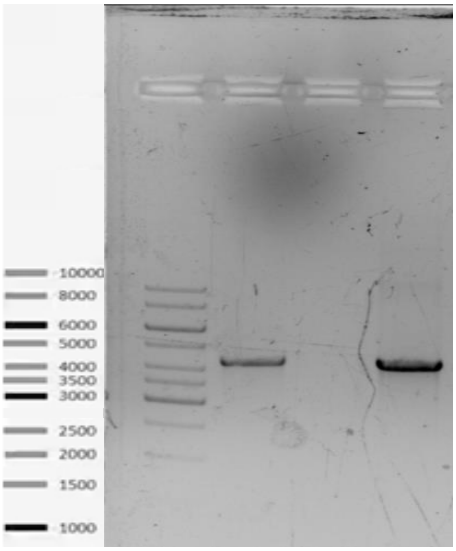

Figure 5: Amplicons of PCR using CEA10*Δku80* to produce tailed *treB* show bands of approximately 4.1kb, as indicated by generuler 1kb plus ladder.

As can be seen in Figure 5, the expected amplicon of 4.1kb was found, indicating proper amplification of TreB with tails complementary to the empty pUC19 plasmid. With XmaI, the empty pUC19 plasmid and the tailed *treB* could be digested, resulting in complementary DNA fragments for cloning. After dephosphorylation of the vector and successfully cloning the *treB* into the plasmid, the K.I. plasmid and a previously prepared PFCC322 plasmid could be cotransformed into a *ΔtreB* mutant strain for complementation. However, digestion of the pUC19 plasmid and tailed *treB* has yet to be performed due to unexpected issues related to DNA extraction. Therefore, the digestion and cloning of the tailed *treB* into the plasmid using XmaI, and ultimately the creation of a K.I. strain of an *A. fumigatus ΔtreB* mutant must be performed in future experiments.

3.2. Chemical and genomic inhibition of TreB

Inhibition of TreB was performed through the exposure to validamycin A using a EUCAST protocol, and OD600 was measured after 24 and 48 hours of incubation (see Figure 13 and Figure 14 in Appendix 1: supplementary figures). Unfortunately, no clear correlation between validamycin A concentration and OD600 was observed after both 24 and 48 hours of incubation for RPMI 1640 and AMM containing 20mM glucose. Considering the germination dynamics in AMM containing 20mM trehalose, a negative correlation for OD600 with validamycin was observed at 2 mg/mL validamycin A or higher for *A. niger* (Fig 5A) and at 128 mg/mL or higher for *A. nidulans* (Fig 5B) but not for *A. fumigatus* CEA10 and CEA10*Δku80* (see Figure 6C and D). However, growth of CEA10 in AMM with trehalose is rather low, yet no additional inhibition with validamycin was observed. When the carbon source in AMM was replaced with proline, such correlation between OD600 and validamycin A could not be observed for any *Aspergillus* species (see Figure 15 in Appendix 1: supplementary figures).

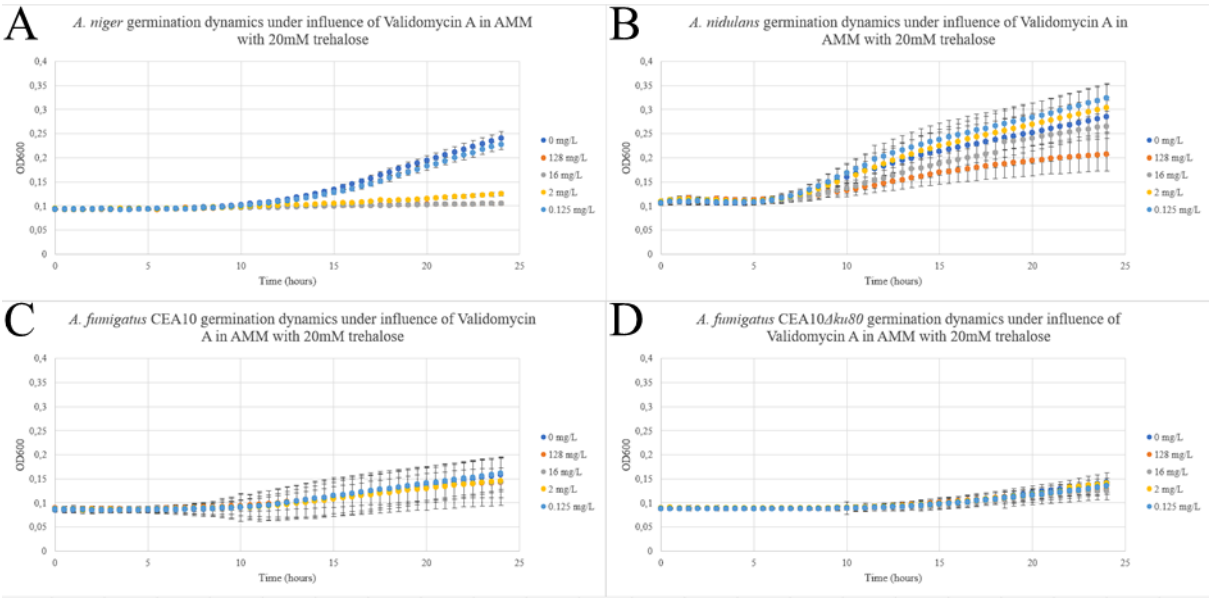

Figure 6: Germination dynamics in AMM with 20mM trehalose shows inhibition of spores of *A. niger* (A) and *A. nidulans* (B), but not *A. fumigatus* (C and D). Validamycin A concentrations 0 mg/L (dark blue), 0.125 mg/L (light blue), 2 mg/L (yellow), 16 mg/L (grey) and 128 mg/L (orange) were tested. Error bars represent standard error of the mean (N=3).

Simultaneously, an attempt was made to create a *ΔtreB* mutant of *A. fumigatus* CEA10*Δku80* using a protoplast mediated CRISPR/Cas9 approach. Hygromycin B resistant colonies with varying morphologies were recovered. The hygromycin B resistant colonies were purified twice using small GMMA plates containing 100 µg/mL hygromycin B, and label 1B-14B.

First, the potential candidates 1B-14B and CEA10*Δku80* were cultured without stresses on either liquid RPMI 1640 with 20mM glucose, or liquid AMM containing 20mM trehalose, proline or glutamate, and the OD600 was measured every 30 minutes for 24 hours (Figure 7). Although performed only once, no clear differences in growth curves of strains 1B-14B in RPMI 1640 nor any AMM variant at OD600 was observed as compared to the wild type strain CEA10*Δku80* (Figure 7, red dots), since the candidate strains did not consistently grow more or less over all four media than the CEA10*Δku80* control.

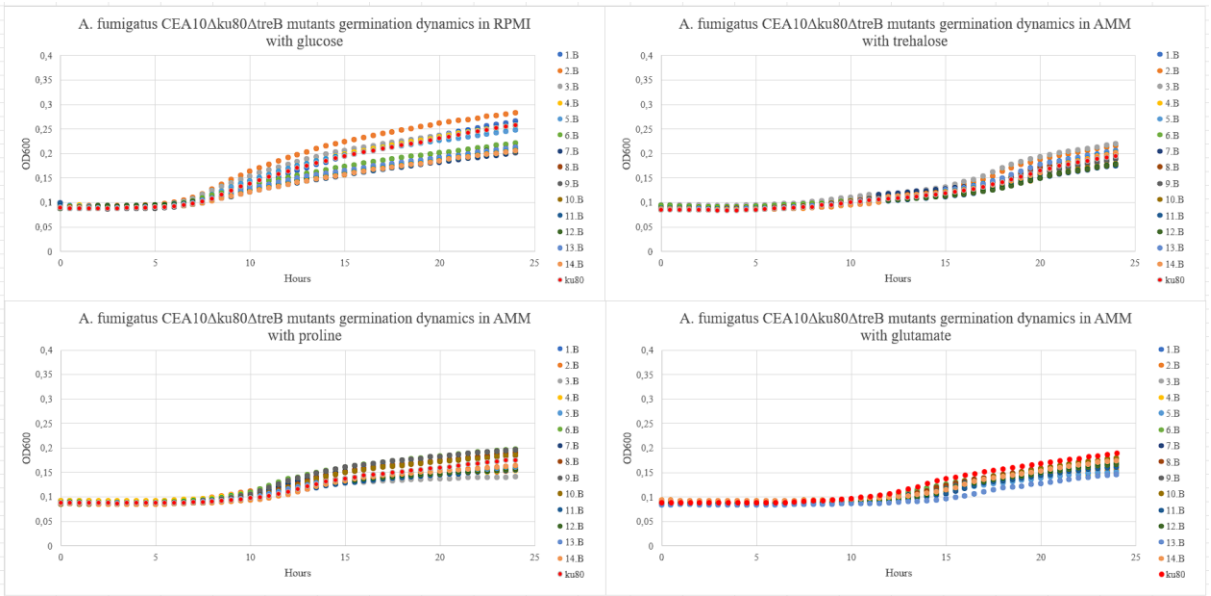

Figure 7: Germination dynamics of *A. fumigatus* CEA10*Δku80* and potential *ΔtreB* mutants 1B-14B in RPMI 1640 with 20 mM glucose, AMM with 20mM trehalose, proline or glutamate show little to no differences between strains (N= 3).

The microwells of these cultures were also checked visually through microscopy, to check whether the homogenous germination kinetics is linked to an equally homogenous micromorphology. As illustrated in Figure 8, morphological differences did seem to appear between the wild type CEA10*Δku80* and candidate strains when these strains were cultured in AMM containing trehalose (Figure 8 B and D) and proline (Figure 8 C and D). For growth in AMM containing trehalose, the candidate strains appeared to grow more densely than its non-transformed counterpart with seemingly thinner hyphae. Interestingly, semi-germinated spores were mostly observed for candidate strains in proline, unlike the CEA10*Δku80* strain.

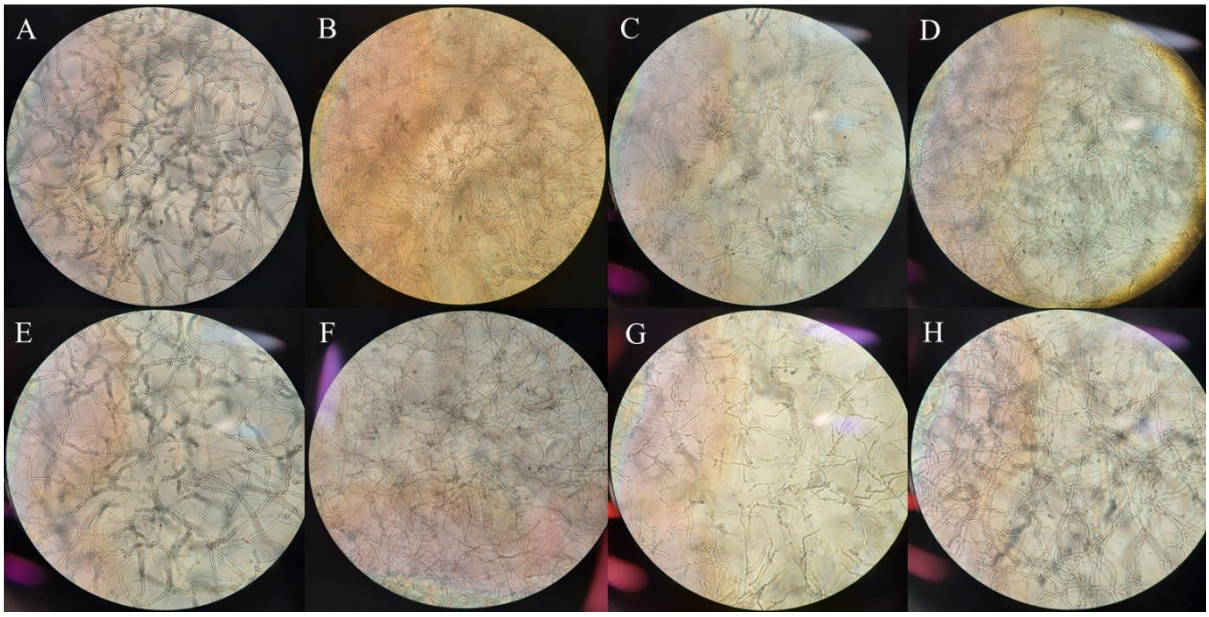

Figure 8: *A. fumigatus* CEA10Δku80 germlings (A-D) and A. fumigatus candidate 10B (E-H) in RPMI 1640, AMM with 20mM trehalose, proline or glutamate (left to right).

To further screen for successful *ΔtreB* candidates using phenotype screening, heat-shock experiments were conducted with spore suspensions of the candidate strains 1B-14B and CEA10*Δku80*. During this heat-shock experiment, 100 µL of AMM spore suspension (250 spores/mL) were exposed to either 55°C for 20 minutes immediately after suspending the spores, or at 50°C for 20 minutes after 3 hours pre-incubation of the AMM spore suspension. The heat-shocked spore suspensions were plated on GMMA plates and colonies were counted per plate. The heat resistance (see Table 3)was expressed in the R-value, as earlier described in Equation 1. Although only performed once, it has been found that strain 1B, 5B, 7B and 9B-12B showed an R-value greater than 1, unlike the CEA10*Δku80* control (R= 0.591). These findings might be a first indication of an altered phenotype for stress-resilience.

Table 3: the ratio CFU of direct ($n_{0h,\ 55°C}$) and indirect ($N_{3h,\ 50°C}$) heat-shock show indications of altered phenotype regarding stress tolerance.

Strain	$n_{0h,\ 55°C}$	$N_{3h,\ 50°C}$	R
1B	8	13	1.625
2B	26	17	0.654
3B	21	19	0.905
4B	19	9	0.474
5B	11	42	0.3818
6B	28	27	0.964
7B	61	68	1.115
8B	32	19	0.594
9B	24	28	1.167
10B	22	25	1.136
11B	25	35	1.400
12B	21	24	1.143
13B	15	11	0.733
14B	42	33	0.786
CEA10*Δku80*	88	52	0.591

K.O. screening was expanded by growing $1*10^6$ spores for 3 days at 37°C on non-stress agar media, namely RPMI 1640, 5% YPDA and GMMA, and GMMA stress media, which include 2M NaCl, HCl (pH= 3), NaOH (pH= 8) or Congo red (25 µg/mL, Figure 9). As expected, all strains showed complete coverage of the agar plates for RPMI 1640 and 5% YPD and almost complete coverage over the GMMA plates. Even though this experiment has been performed only once, this overview strongly suggests that 2M NaCl and 25 µL/mL Congo Red inhibit *A. fumigatus* candidate strains 1B-14B and wild-type CEA10*Δku80* the most, while addition of HCl (pH= 3) only moderately inhibits the tested strains. Compared to the CEA10*Δku80* strain, no differences in micromorphology, such as colony size, shape or pigment was found. This indicates the absence of *ΔtreB* mutants.

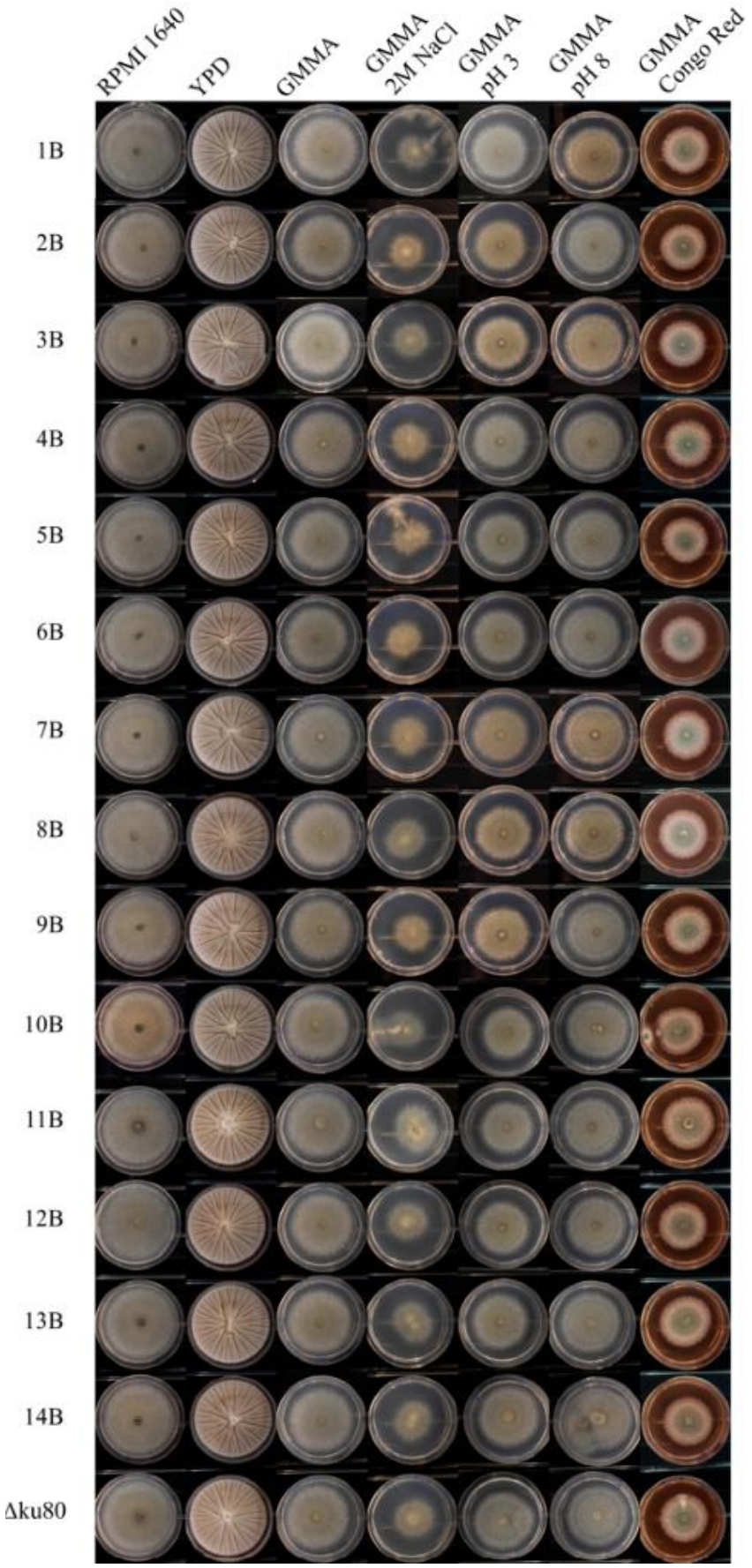

Figure 9: Overview of photographs taken 3 days after inoculation shows no alterations in colony morphology of the candidate strains relative to untreated *A. fumigatus* CEA10Δku80 spores.

To prove that the observed phenotype is indeed linked to a wild-type genotype, direct colony PCR was performed by boiling 30 µL of spore suspension at 95°C for 15 minutes, followed by snap freezing at -80°C for 10 minutes. After thawing, 5 µL sample was used for PCR as described in paragraph 2.2.2 of the Materials and methods. Neither 4kb (wild-type genotype) or 900bp (*ΔtreB* genotype) bands were observed on a 1% agarose gel (data not shown) for the PCR reaction of both candidate strains and the CEA10*Δku80* strain using the direct colony PCR approach, indicating unsuccessful amplification of the directly isolated DNA. By growing *A. fumigatus* candidate strains for days in TM, followed by harvesting of mycelium, freeze drying and DNA extraction using a DNeasy DNA extraction kit, proper DNA template seemed to be produced as PCR reactions with these templates did result in visible bands (Figure 10). Indeed, the lack of difference in phenotype as shown in Figure 9 was due to a wild-type genotype for 1B-6B, 9B, 10B, 13B and 14B. Unfortunately, no bands were visible for the remaining strains 7B, 8B, 11B and 12B.

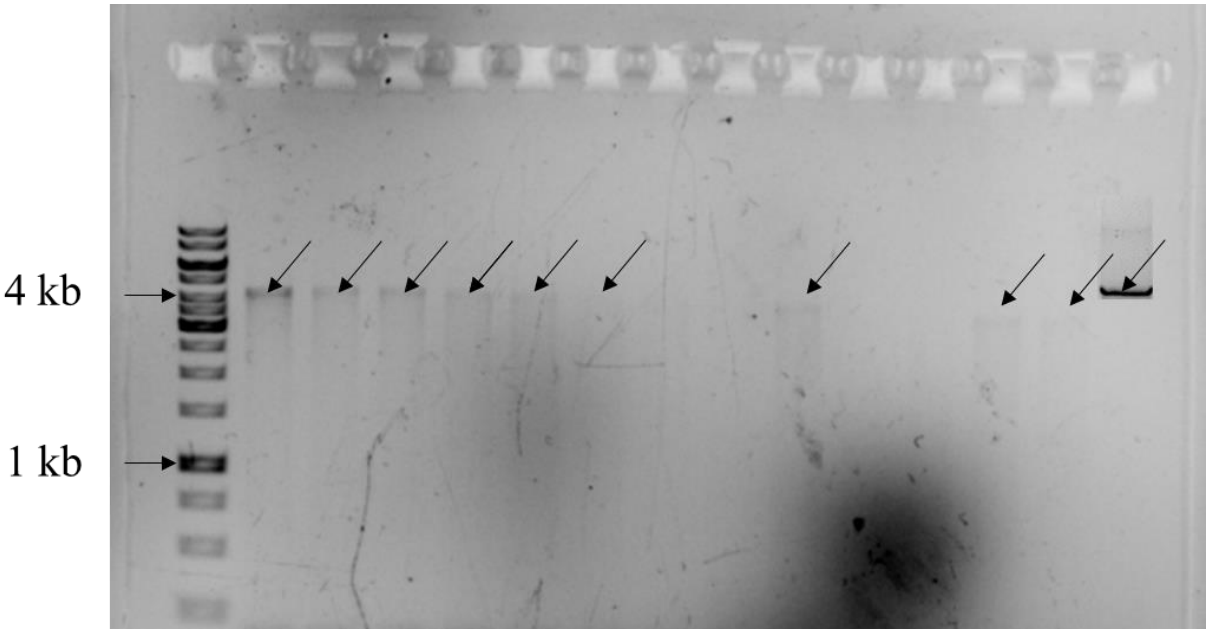

Figure 10: Performing PCR with the 34E Forward and 37E Reverse primers show amplicons of approximately 4 kb in accordance with the expected amplicon size as indicated by the black arrow, suggesting the presence of the complete TreB gene (from left to right: 1B-9B, 11B-14B and 10B).

3.3. Active TreB production and extraction

For the design of TreB-inhibiting peptides, a pET52 plasmid carrying the exons of *treB* was successfully transformed into chemically competent *E. coli* BL21 cells. The system was initially checked by immediate boiling of IPTG-activated *E. coli* and controls for 10 minutes in laemmli buffer. After running these samples on an SDS-PAGE gel, a clean band was observed at approximately 85 kDa (Figure 11).

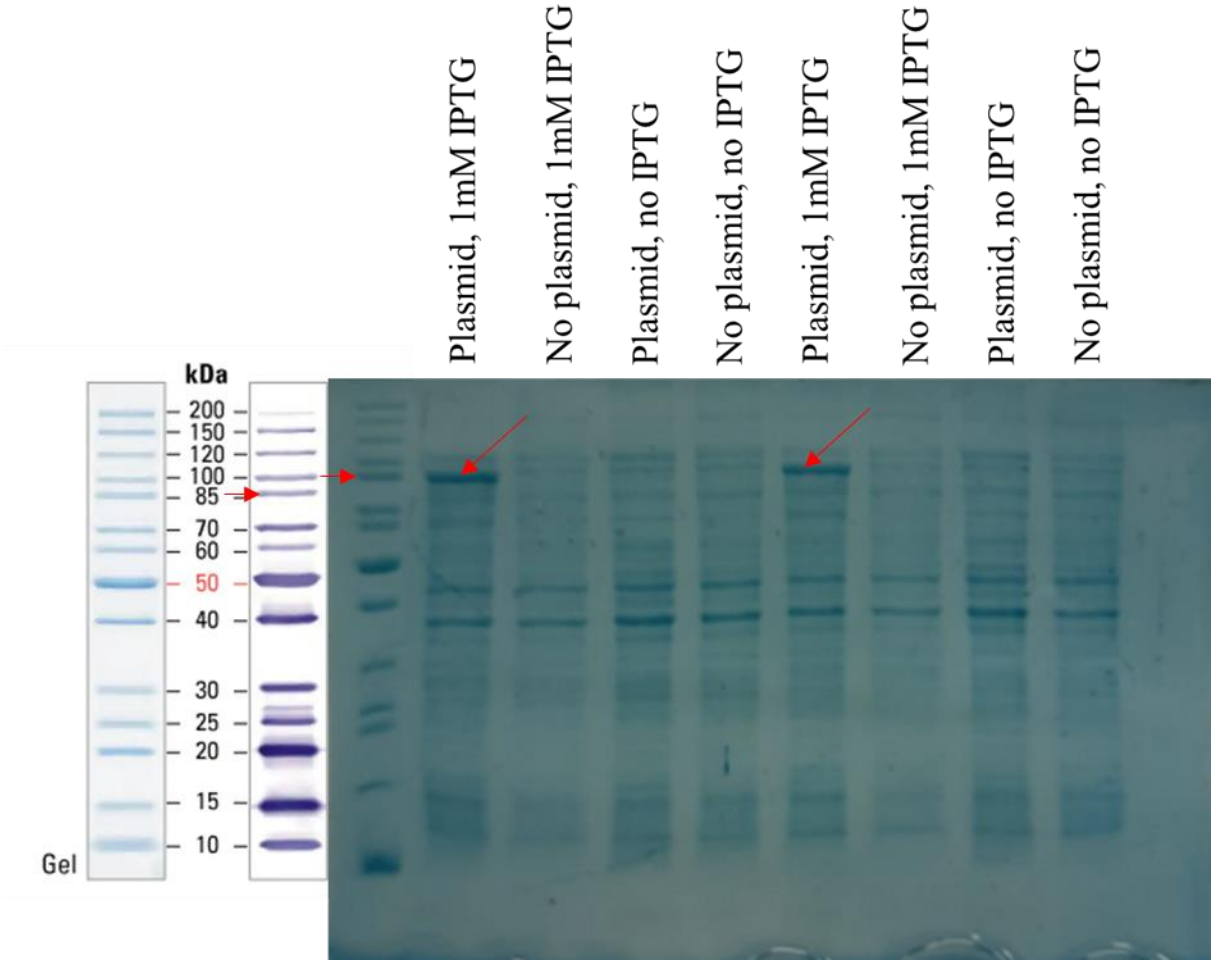

Figure 11: An SDS-PAGE gel shows a thick, but tight band present around 85 kDa (marked with red arrows). As TreB was expected to have 86654Da and other conditions like the lack of transformed plasmid and/or IPTG, this must be the target protein.

As this finding overlaps with the expected size for active TreB, protein expression through IPTG activation and purification using a Ni-NTA column were performed. However, the amount of protein purified remained very low. To confirm the absence of TreB, the resulting protein samples of the purification were used in a trehalose kit. Indeed, enzymatic activity seems to be absent due to similar absorbances measured for the samples, relative to the positive and negative control (see Table 4 in Appendix 2: supplementary tables).

The low production and absence of trehalase activity might be due to a mutation in the cloned *treB* gene. Therefore, DNA sequencing was performed to check whether the cloned treB gene was intact. PCR products of overlapping parts were generated and sequenced through sanger sequencing. The obtained sequence data confirmed a 4-codon deletion at position 2264 in the gene (Figure 12), which is close to the 10x His-Tag c-terminus. This means the created during IPTG-activation is a TreB protein lacking 4 amino acids on position 754-758. Other than the 4-codon deletion, the sequence data does not seem to point towards any unwanted mutations of the *treB* gene.

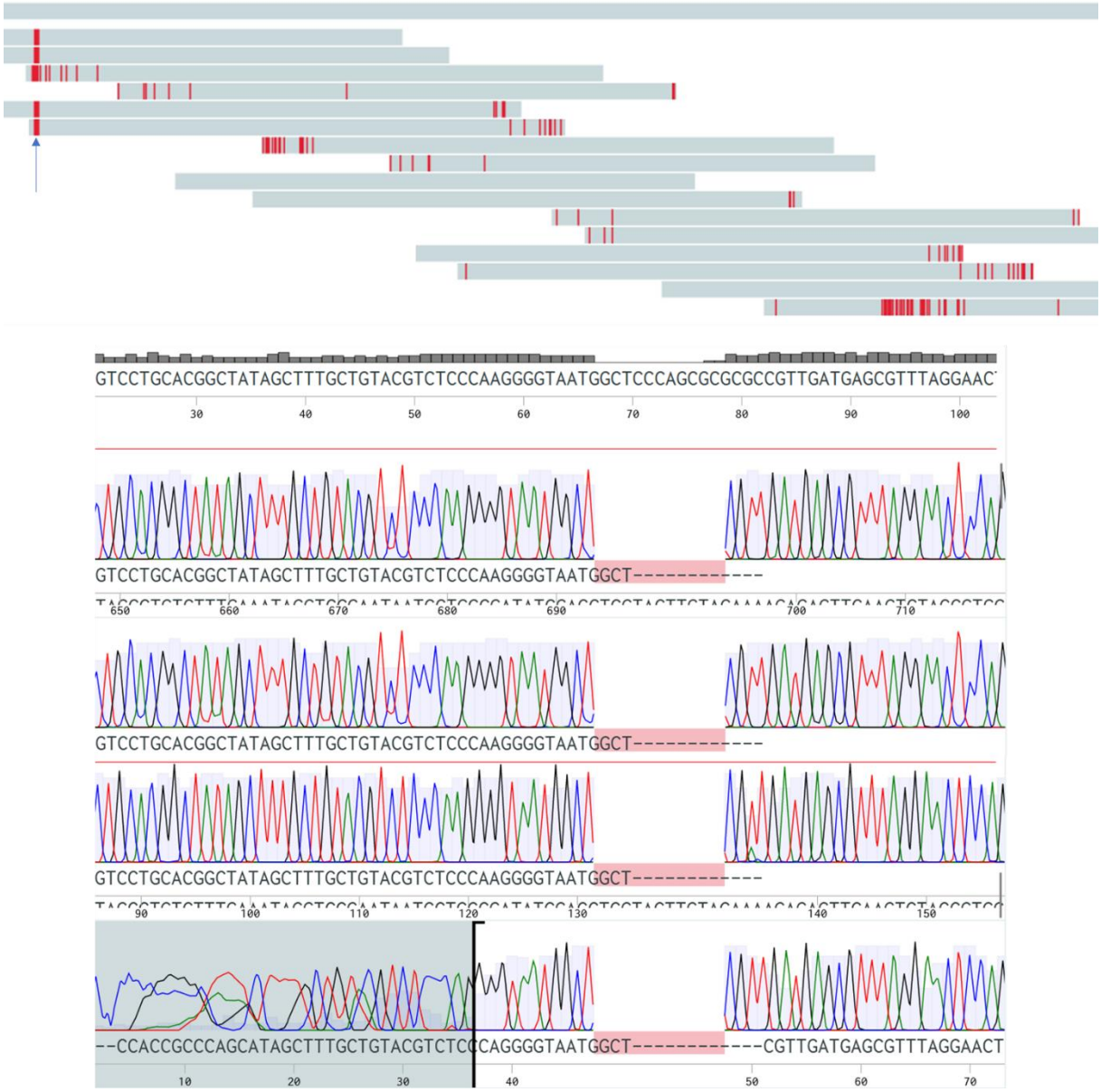

Figure 12: Alignment of the treB gene with sequenced amplicons derived from the *treB* gene shows a clear deletion at position 2264 (marked with blue arrow, gene is oriented from right to left) in the sequenced amplicons using the seq_1_Rev, Ch_1_Rev seq_1_Fw, Ch_1_Fw primers (sequence 2, 3, 6 and 7).

4. Discussion

In this study, trehalose was set as a novel antifungal target for aspergillosis treatment and management. For this, active trehalase had to be produced for selection of new inhibitory peptides via the RaPID Bioaffinity assay [31]. Even though the transformed BL21 *E. coli* did show production of an 85 kDa protein after IPTG activation and protein purification, the amounts remained very low. Furthermore, the absence of enzymatic activity was demonstrated with the trehalose kit. This strongly indicates that the purified protein sample does not contain (active) *A. fumigatus* trehalase. As active neutral trehalase has been expressed for *Neurospora crassa* (*N. crassa*) in *E. coli* BL21, the lacking post-translational modification is most likely not the cause of no TreB activity [43]. In addition, the *N. crassa* TreB was extracted from the Ni-NTA column using a lower concentration of imidazole, excluding the possibility of the His-tagged TreB to be stuck on the Ni-NTA column. Therefore, a possible mutation in the cloned *treB* gene might be responsible. By checking DNA sequence data of the used pET52 plasmid containing the *treB* gene, a 12 bp in-frame deletion was found starting at nucleotide 2264 which results in the absence of 4 amino acids at position 754 to 758 (Figure 12). The absence of four amino acids might be due to an error in the synthesis, as the *treB* expression gene was produced by a third party. Furthermore, the absence of the four amino acids might explain the low expression or stability of the protein as well as absence of enzymatic activity. Absence of four amino acids might result in a misfolded TreB protein lacking any enzymatic activity. It is not clear how the absence of four amino acids would affect the conformation of *A. fumigatus* TreB, because three-dimensional structures of fungal neutral trehalase have only been elucidated for *Saccharomyces cerevisiae* (*S. cerevisiae*) [44]. By checking the three-dimensional structure of *S. cerevisiae* neutral trehalase (PBD: 5JTA) in YASARA (version 22.9.24), it becomes clear that the last amino acids of the protein are either part of alpha-helix structures or interact with the hydrophobic backbone of these alpha-helixes (Figure 16, Appendix 1: supplementary figures). This means the absence of 4 amino acids in *A. fumigatus* neutral trehalase could disrupt the formation of alpha-helixes or the interaction herewith. In addition, Alphafold suggests amino acids 754-758 to be part of an alpha-helix structure in *A. fumigatus* TreB (see Figure 17, accession: Q4WQP4), highlighting the potential instability and alternative three-dimensional structure of the TreB produced in this study, despite this part of the protein not being part of the substrate-specific binding site. Redoing the transformation with new unmutated TreB should be done in the future to progress this element of the project. This could be done by integrating the 12bp deletion in two primer sets and amplifying the gene with these primers. By cleaning up the newly created amplicons, a fusion PCR could be performed to fuse the overlapping sequence of the amplicons, creating a recovered gene ready for digestion and cloning. This will require redoing the digestion using NotI of the empty pET52 plasmid and the recovered *treB* expression gene. This must then be followed up by ligating these two products after dephosphorylation of the vector. Transformation could then be performed in NEB10 *E. coli* cells to create more successfully cloned plasmid and consequentially be able to redo the transformation and expression of *treB* in BL21 *E. coli*.

Furthermore, an attempt was made to create a *ΔtreB* mutant in *A. fumigatus* CEA10*Δku80*. As growth was observed on the selection media containing 100 µg/mL hygromycin B, colonies were selected and purified successfully on purification plates containing 100 µg/mL hygromycin B as well. These 14 different candidates, named 1B-14B, were grown in different liquid media, but did not seem to grow differently compared to the untreated *A. fumigatus* CEA10*Δku80* strain. Regardless of the observed germination kinetics, the micromorphology of candidate strains did seem to differ, like less germination in AMM with proline, and the formation of thinner hyphae in AMM with trehalose. Heat-shock experiments gave indications that some of the strains might be *ΔtreB* mutants due to an increase of heat resistant spores. However, this change in phenotype – such as change in pigmentation, colony size and shape – was not found when spores were grown on an array of non-stress agar media and GMMA containing 2M NaCl, HCl (pH= 3), NaOH (pH= 8) and Congo Red (25 µg/mL). Considering the observed phenotype of the candidate strains, it was suspected that no *ΔtreB* mutants were isolated.

Indeed, performing PCR of the *treB* gene in the candidate strains did confirm absence of the *ΔtreB* mutant genotype.

As the candidate strains did show a wild-type genotype, it is unclear as to why the changes in micromorphology did occur. One possible explanation could be the mere exposure to the protoplast mediated CRISPR/Cas9 protocol, since unexpected alterations in growth and morphology have been documented in other studies [45], [46]. Additionally, the genotype screening strongly suggests insufficient selection pressure on the selection plates containing hygromycin B, as wild-type genotype *A. fumigatus* was apparently able to form colonies. This could be explained by the age of the hygromycin B stock that was used in this study, meaning less hygromycin B was present in the selection plates than calculated. As the transformation itself has been performed only once, the protocol must be repeated in the future with a fresh hygromycin B stock and – if possible – optimized for *A. fumigatus*. Naturally, a K.I. transformation must be performed with the *ΔtreB* mutant strain hereafter to conclude that the changes in phenotype are exclusively caused by the deletion of *treB*.

Moreover, *Aspergilli* phenotypes were analysed after exposure to validamycin A, a chemical compound capable of inhibiting TreB enzymatic activity [30] [47]. Unlike previously conducted research, the resistance of validamycin A in this study was based on a spectrophotometric approach, like the standardized EUCAST protocol.

In RPMI 1640 media or AMM with 20mM glucose, there did not seem to be an inhibiting effect of validamycin A on any of the tested *Aspergillus* species. This absence of inhibition was also observed when fungi were grown in AMM with proline.

It was found that the tested *Aspergillus* species did not show lessened growth or delayed germination due to validamycin A in RPMI 1640 media or AMM with 20mM glucose or AMM with 20mM proline. The lack of inhibition by validamycin A could be due to the presence of carbon nutrients other than trehalose, such as 20 mM glucose in RPMI and AMM or 20mM proline in AMM, which allow the germinating spores to bypass the bottleneck created by the TreB inhibition. Indeed, it was previously found that mannitol could serve as an alternative intracellular carbon source to trehalose and carbon transporters become active within the first hour after breaking dormancy [21] [48]. Therefore, testing validamycin A in rich media like RPMI 1640 give inconclusive results regarding the antifungal effects (Figure 13). Media that force the metabolism of trehalose might have to be used to elucidate the full antifungal effect of validamycin A. The amino acid proline was expected to act as a carbon source and is for example available in the human lungs [49]. Remarkably, growth was very limited in presence of proline as compared to glucose (Figure 7). Interestingly, inhibition of germination was found *A. niger* and *A. nidulans* but not *A. fumigatus* during growth in AMM containing trehalose. Even though it is not clear why *A. fumigatus* does seem to grow regardless of the validamycin A concentration, the inhibition of *A. niger* and *A. nidulans* does demonstrate how the overall germination is limited, but not delayed. Germination of *A. fumigatus* in AMM with trehalose is rather limited as compared to *A. niger* of *A. nidulans*. It suggests that external trehalose is more difficult to be processed with the external trehalase compared to extracellular glucose [50]. Yet, no further reduction in growth was observed with validamycin A for *A. fumigatus*, in contrast to *A. nidulans* and *A. niger*. It is not clear why germination of *A. fumigatus* is not affected by validamycin A. Possibly, the active site is somewhat different resulting in absence in trehalase inhibition. However, it might also be that *A. fumigatus* trehalase is inhibited, but that this inhibition does not result in delayed germination due to some alternative systems supporting germination. To unravel this point a *ΔtreB* strains is required to investigate if alternative systems rescue germination. If not, it opens a way to the clinical setting, this means a hypothetical TreB inhibiting peptide as prophylaxis could reduce germination of the *Aspergillus fumigatus* spores.

5. Conclusion

In conclusion, the role of TreB in *A. fumigatus* spores has been explored in this study. Although preliminary, inhibition of germinating spores through blockage of TreB has demonstrated for *A niger* and *A nidulans* through the application of validamycin A but not for *A. fumigatus*. The incomplete development of germinated spores may hold potential for medical application as non-germinating spores could lead to clearance by the immune system and improper colonization of the lungs, excluding the manifestation of IPA and other severe forms of aspergillosis alike. To achieve this, more research must be done, such as CRISPR/Cas9 simulations and obtaining active TreB.

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Appendices

Appendix 1: supplementary figures

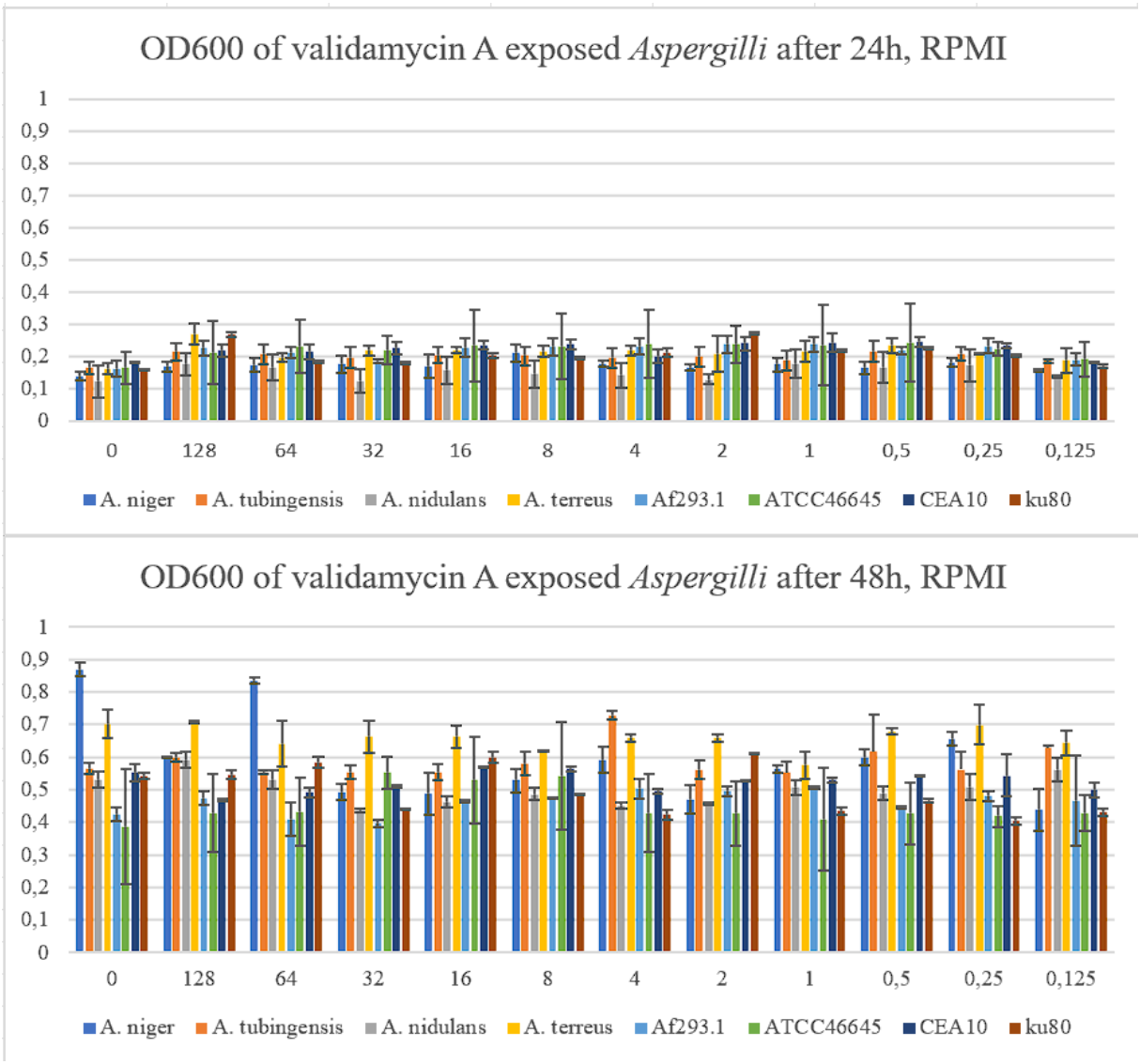

Figure 13: OD600 values of *Aspergilli* (strain numbers are *A. fumigatus*) measured after 24 and 48 hours in liquid RPMI 1640 medium, containing 0-128 ug/mL validamycin A. Error bars represent standard error of the mean (N=3).

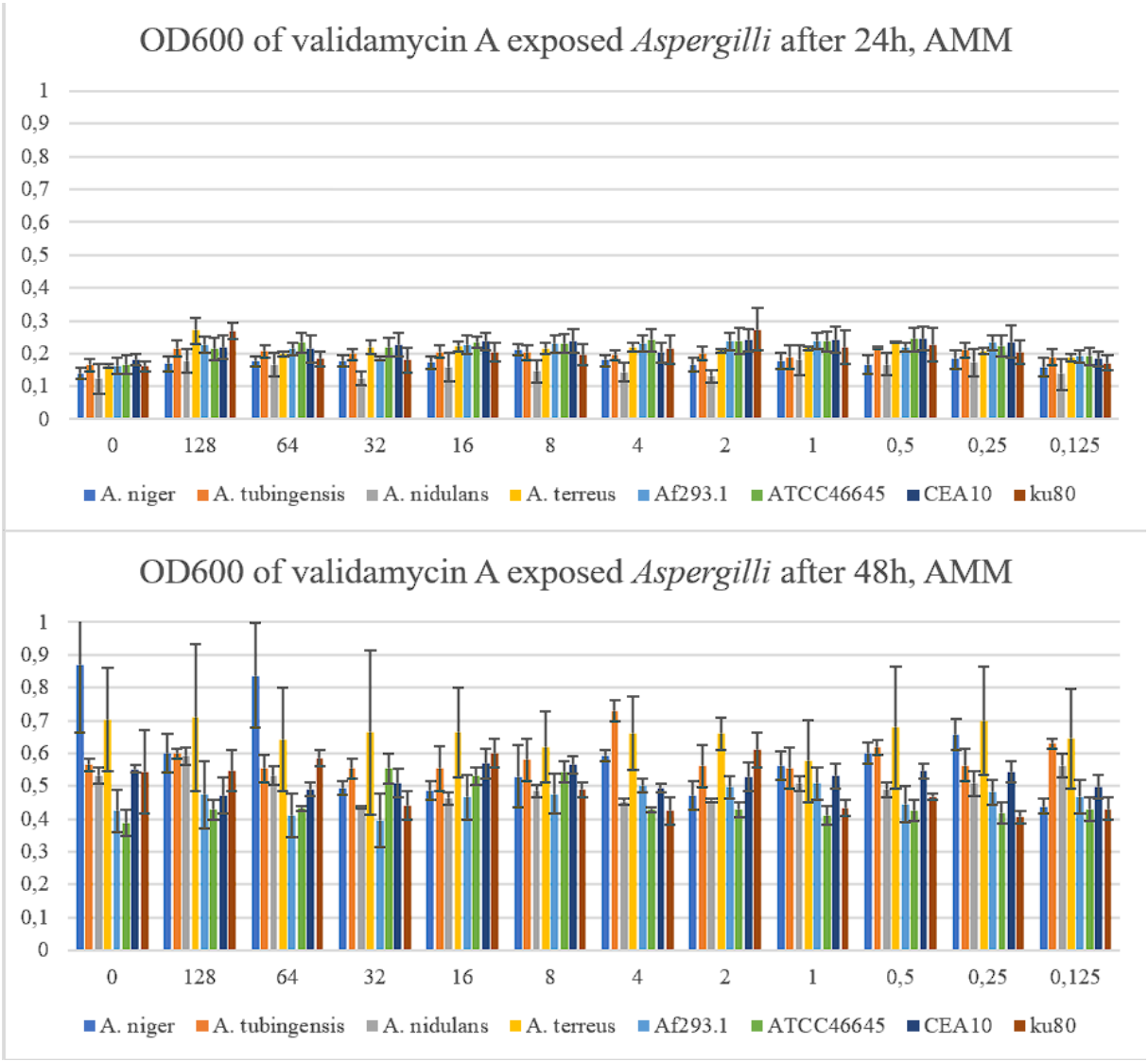

Figure 14: OD600 values of *Aspergilli* (strain numbers are *A. fumigatus*) measured after 24 and 48 hours in liquid RPMI 1640 medium, containing 0-128 ug/mL validamycin A. Error bars represent standard error of the mean (N=3).

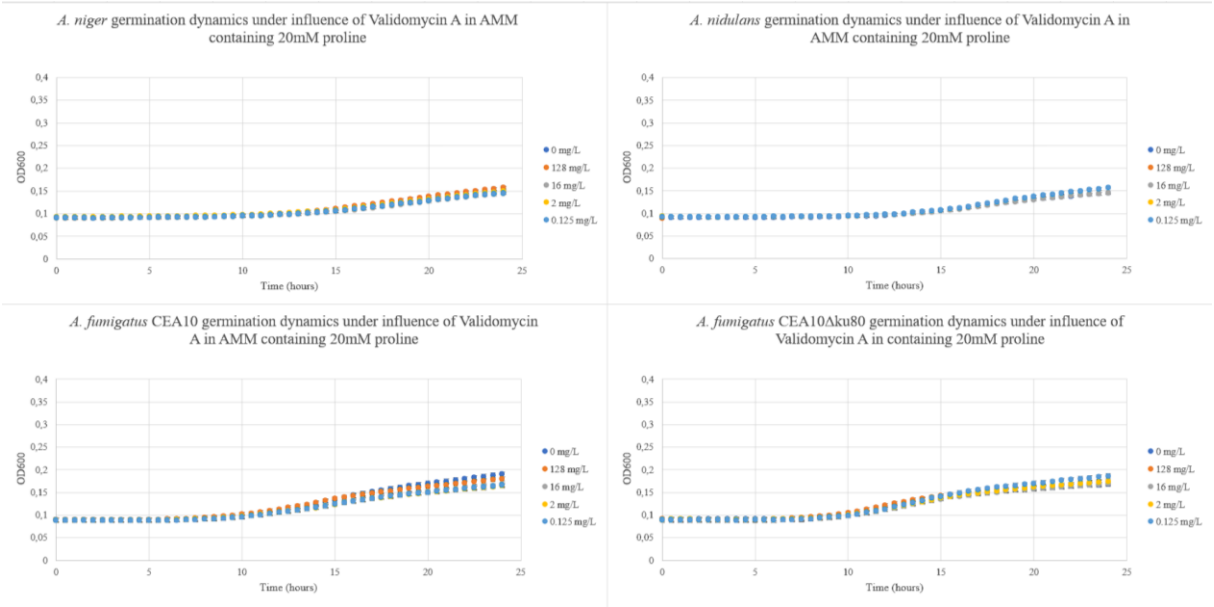

Figure 15: Germination dynamics in AMM with 20mM proline shows no increased inhibition of spores when the validamycin A concentration increased. Validamycin A concentrations 0 mg/L (dark blue), 0.125 mg/L (light blue), 2 mg/L (yellow), 16 mg/L (grey) and and 128 mg/L (orange) were tested. Error bars represent standard error of the mean (N=3).

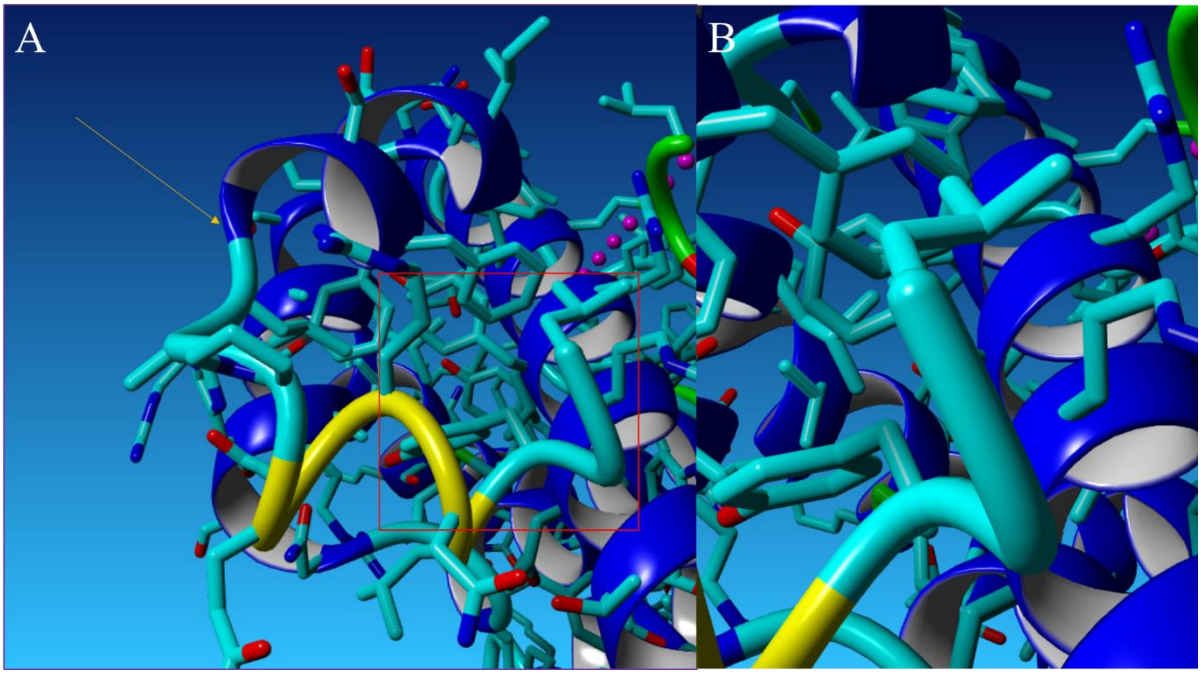

Figure 16: Analysis of S. cerevisiae neutral trehalase (PDB: 5JTA) shows that the last 10 amino acids (A, amino acid 741 indicated by the orange arrow) are either part of an alpha-helix or have hydrophobic interactions with surrounding alpha-helixes (B, zoomed in red marked region in A).

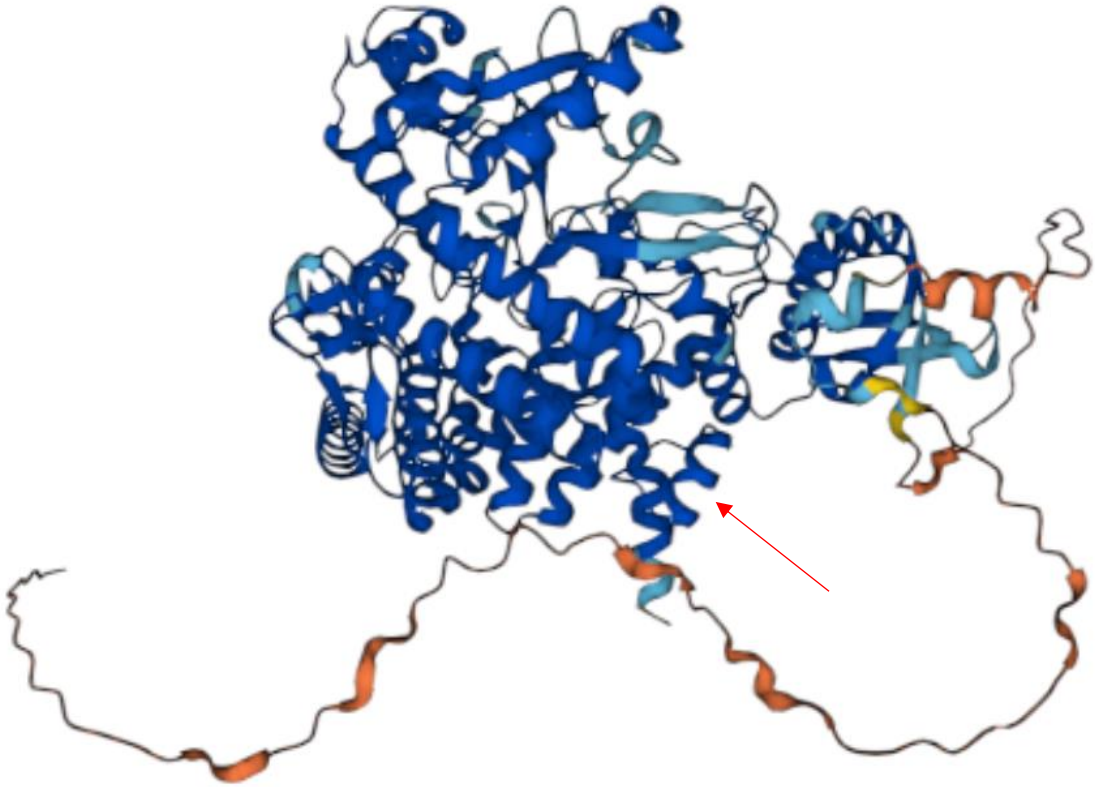

Figure 17: The Alphafold predicted model of A. fumigatus TreB reveals that amino-acid 754-758 participate in an α-helix structure (indicated with the red arrow), potentially playing a key role in the chemical stability of the protein.

Appendix 2: supplementary tables

Table 4: results of the trehalose kit show a lack of enzymatic activity of samples, contributing to the suspicion of the target protein being absent in the ultimate protein samples.

Sample	Absorbance (340nm)
Negative control	0.129
Positive control	1.239
Sample (storage -80°C)	0.134
Sample (storage -20°C)	0.132