Cheating and cooperation in Aspergillus niger

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Major Internship Thesis
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Foreword

Hereby I present my major internship thesis that includes all of my ideas, concepts and findings that I have gathered during my internship at the Utrecht University microbiology department. Here, academics are working on fungi to uncover fundamental questions, trying to fill in the many knowledge gaps that surround these microorganisms. I'm grateful that I have been part of the fungal biotechnology group, led by Han Wösten where I have worked on cheating and cooperation in Aspergillus niger. I appreciate the help of my daily supervisor Jun Lyu who had the patience to see me progress throughout these nine months and who was always interested in fresh ideas which really helped me in making this project my own. Furthermore, I would like to thank all other academics that I have met that gave me advise and offered help when needed.

Lay summary

In research, scientists are eager to find answers to the unknown, to optimize processes and to come up with solutions to aid in human's current challenges. In doing so, it is important to keep in touch with nature, since we extract its resources for our own goods. One particular part of nature that is slowly getting more recognition is the world of fungi.
Even though fungi are found on all continents of the world, research on fungi is more dispersed compared to other microorganism research fields, which is why fungi were for a long time still believed to be plants. However, they are more related to higher life such as animals and play fascinating but complex roles in nature. Fungi are generally regarded as the recycling machines of nature, turning decaying organic matter into nutrients to be taken up by the living world. They ultimately close the ecological circle of life. Unfortunately most fungi grow for the most part underground and out of sight which makes them hard to study. Some will produce mushrooms, which are the "fruits" of the mycelial network. Others are more commonly visible, growing as molds on food. Probably the most successful fungus we know of are these that belong to the genus of Aspergillus. In biotechnology, many aspergillus species are being used as cell factories, producing specific compounds that can be extracted when grown on a big scale. Specifically Aspergillus niger (*A. niger*) is known for its variety of enzymes that it secretes and is commercially exploited for numerous molecules such as precursors for pharmaceuticals and food additives. In the process of optimizing enzyme production in *A. niger*, researchers found that its morphology influences how well it produces the enzyme of interest. More specifically, in liquid cultures it grows in colonies, ball-shaped structures that act as a micro population sensing its environment. Whether colonies are big or small changes its enzyme expression. This study wanted to focus on how these different colonies can interact on the fundamental level of cooperation, the most important asset to the success of any organism. Therefore, a system was developed of *A. niger* with a handicap, trying to inhibit its growth on sucrose, a simple sugar. Furthermore, another available mutant of *A. niger* was applied to a set of experiments to test whether if it's possible to standardize an assessment for interactions between *A. niger* and a mutant. Hopefully, the mutants of the sucrose-system can be applied to these experiments once it is completed. This could greatly improve our understanding of cooperation and cheating in *A. niger.*

Abstract

Aspergillus niger has established itself in industry as a cell factory and in research as an model organism. Yet, much remains to be known about the mechanisms it uses to select for cooperation or against cheating. This study aimed to develop a sucrose-system, using sucrose mutants as a proof of concept to test a set of pilot experiments that were in turn developed to make a standardized way of assessing mutant to wild type interactions. Due to time limitations, the pilot experiments were ultimately tested using an available uncharacterized glucoamylase mutant. In literature it is described that high relatedness among individuals lowers the chances of a cheater to incorporate into the germline. Therefore, hypothesis was that since the strains are very related, they would cooperate. The results suggest that the mutant might act as a cheater in liquid cultures.

2. Introduction

In biology, it is possible to study the phenomenon of evolution on all levels of organization. Perhaps the most fundamental organizational aspect being the transition from unicellular to multicellular organisms. This transition draws great interest in that it has occurred many times throughout evolution in all kingdoms, which has kept taxonomists occupied with looking for similarities and to adjudge new species to new subspecies and so forth (Whittaker 1969). In a lot of organisms, transitioning from uni- to multicellular is induced by the environment, which is why under lab conditions this was sometimes overlooked but has now long been regarded as a common attribute to some microorganisms (Branda et al. 2001; Shapiro 1998). This transition of uni- to multicellularity is viewed as a relatively simple one, given that the unicellular ancestors possibly already owned the developmental foundations needed to organize itself from a lower (unicellular) to a higher level (multicellular) organism, explaining the frequency in which it has arisen (Keller and Surette 2006; Miller and Bassler 2001; Michod and Richard 1997).

There are a variety of ways in which multicellularity can occur. One example is the incomplete division of daughter cells, creating a clump of cells all partly physically connected to one another. Stringent clonal development results in low genetic variation among cell lineages and differentiation of individual cells or groups of cells manifests itself in changes in gene expression or post-transcriptional modification, rather than actual genetic differences between cells (King 2004). Additionally in clonal development, genetic variation is diluted throughout the offspring and thus reduces selection for mutated cell lineages as such. Most interestingly, this lowers the evolutionary efficiency of cheater cell lineages that incline their own reproduction over that of the whole organism, since such singular characteristics are not favored in the formation of multicellular propagules (Grosberg and Strathmann 2007). Another example is through aggregation of individual cells that coalesce to form a colony, thus deriving from different cell lineages. How both these merged cell lineages are influenced by genetic diversity plays out on several stages during development. Both ways have their advantages and drawbacks. If we consider it from an evolutionary perspective, where mutations are the driving motor behind optimizing an organism's strategy of survival, clustering of cells from the same lineage is less prone to incorporate negative mutations of an individual cell, whereas clustering through aggregation there's higher chances of cell lineages merging that have a lower fitness. This may be the reason why in most obligatory aggregating organisms, the level of differentiation is lower compared to clonally developing organisms (Bonner 1998). For cells cooperating to establish this multicellularity, complex communication systems have to be set up, which in turn opens up possibilities for cheater-cells to capitalize on the cooperating cells. These mechanisms differ from the level of cooperation they apply to, whether within organism- or between organism-cooperation.

One possible way to distinguish friend from enemy is self-referent phenotype matching. Here, organisms compare their own phenotype to that of another individual (Holmes and Sherman 1982). Even though animals can learn to recognize kin, this is a trait that can be lost after which self-matching is then used to relearn this skill (Mateo 2010). An example is the use of cuticular hydrocarbons in crickets. Females have many mating individuals so they use these chemicals to mark males after mating and can thereby recognize by their own label which males they have already mated with (Thomas and Simmons 2011). Fungi have also evolved on chemotropism that consists of both chemical attraction and avoidance which ultimately leads to the fine tuning of hyphal orientation (Turrà et al.

2016). Chemical attraction induces sexual hyphal fusion and vegetative fusion. Hyphal fusion is regarded sexually when hyphae of individuals fuse that are contrasting genetically and in mating type. Vegetative fusion can be engaged between related and distinct hyphae (Leeder, Palma-Guerrero, and Glass 2011). The most extensively studied sexual chemotrophy is the mating system of Saccharomyces cerevisiae (S. cerevisiae) and this pheromone based mechanism is highly conserved among ascomycete fungi (Turrà et al. 2016). Considering *Aspergilli* however, this genus of fungi seems to exhibit a high occurrence of heterokaryon incompatibility, which is when contacting hyphae of different mycelial networks exterminate the formation of a heterokaryon and thus blocking sexual hyphal fusion. It is theorized that this is a means of *Aspergilli* to recognize the other individual and to protect itself from incorporating cheaters and this is regulated at the genetic level (Turrà et al. 2016; van Diepeningen et al. 2009).

When recognition of self from non-self, transitions from chemicals such as pheromones to recognizing another's genetics, this is termed allorecognition. Gonçalves and colleagues have identified genes that assess the genetic compatibility between individuals of *Neurospora crassa* (*N. crassa*) upon cell wall contact. Mutations in these genes dissolved the blocking of hyphal fusion between incompatible strains, thereby exposing how intricate the mechanisms behind social cooperation have developed in fungi (Gonçalves et al. 2019). Interestingly, *A. niger* is generally regarded as a presumably asexual species. Since the genes responsible for heterokaryon incompatibility occur during sexual crossing, they have yet to be characterized in *A. niger*. This does not mean that *A. niger* is a completely solitary fungus. *A. niger* has been co-cultured with a hypercellulolytic Trichoderma reesei (*T. reesei*) mutant on sugarcane bagasse that led to synergistic interactions resulting in a major increase of enzyme production in the hydrolysis of the cellulosic biomass (Gutierrez-Correa et al. 1999). Furthermore, *A. niger* has been mixed with Aspergillus terreus (*A. terreus*) on a substrate of banana peels. Again, the co-culture resulted in higher titers for the desired plant cell wall degrading enzymes compared to the mono-cultures (Rehman et al. 2014). Additionally, *A. niger* has been mixed with S. cerevisiae for improved pectinase production at high temperature as well as for the production of raw starch degrading amylases (Zhou, Ge, and Zhang 2011; Abu 2005). Moreover, a mixed culture of *A. niger* with Aspergillus oryzae (*A. oryzae*) degrading wheat bran resulted in a broader spectrum of carbohydrate active enzymes compared to the mono-cultures (Benoit-Gelber et al. 2017). This shows that besides *A. nigers* constraints to exhibit sexual hyphal fusion, it clearly takes on a cooperative lifestyle when co-cultured with other fungi that results in higher titers of desired products.

However, to the best of our knowledge, co-culturing *A. niger* with another *A. niger* mutant strain has not been reported. Therefore this research set out to explore what within-species interactions take place in *A. niger* and a sucrose deficient strain. The expectations of such research, reflecting on the previously discussed literature, were that such interactions would be cooperative rather than competitive. To test the hypothesis, two research lines were explored. The first line of research was to manipulate a wildtype (WT) lab strain to deliberately give it a disadvantage growing on sucrose in standard lab conditions. The second research line was setting up pilot experiments that could then be used to make assessments of the cooperative interactions when mixing such a mutant with its parental WT strain. The initial plan was to utilize these experiments to test the sucrose system but ultimately, a glucoamylase mutant was used in testing the pilot experiments given that the sucrose system had not been completed.

3. Methods

3.1 Media, strains and maintenance

Strains of Escherichia coli (*E.* coli) were cultivated at 37°C with shaking set at 200 rpm in Luria- Bertani (LB), or super optimal catabolite (SOC) medium supplemented 100 µg/ml ampicillin (Amp). All *A. niger* strains in this study (table S3) have N400 (Bos et al. 1988) as background strain. Liquid growth conditions of *A. niger* strains were 30°C with shaking set at 200 rpm. Used medium types consisted of liquid minimal medium (MM), liquid complete medium (CM) or as agar plates. Other solid medium types were potato dextrose agar (PDA), stabilized minimal medium (MMS) and stabilized minimal top-medium (MMST). Supplemented carbon sources were 20 mM glucose (GLU) and sucrose (SUC). In the case of growth experiments, used carbon sources were 5 gr/L GLU or soluble starch. Antibiotics used for *A. niger* were 100 µg/ml hygromycin B (hyg) or phleomycin (phleo). LB medium contains 10 g/L tryptone, 10 g/L NaCl and 5 g/L yeast extract. SOC consists of 5/L g yeast extract, 20 g/L tryptone, 0.584 g/L NaCl, 0.186 g/L KCl, 2.4 g/L MgSO4 and 20 mM glucose. MM contained 6 g/L $NaNO_3$, 1.5 g/L KH_2PO_4, 0.5 g/L KCl, 0.5 g/L $MgSO_4*7H_2O$, 0.2 ml/L Vishniac solution (Vishniac, 1957) with a pH of 6.0. CM is MM but supplemented with 2 g/L tryptone, 1 g/L yeast extract, 0.2 g/L casamino acids, 0.5 g/L yeast ribonucleic acids. MMS is MM with 325 g/L SUC and 15 g/L agar. MMST is MM with 325 g/L SUC, and 6 g/L agar. Stocks of *E. coli* were prepared by adding 200 µl 80% glycerol to 800 µl saturated culture and stored at -80℃. Stocks of *A. niger* were prepared by harvesting spores from a 2 day grown PDA plate into 10 ml of Saline 0.05% Tween-80 solution (ST). This solution was then centrifuged for 5 min at 4000 rpm, the supernatant discarded and 1 ml of glycerol medium (1 part glycerol 87%, 2 parts 1% peptone) was added. This suspension was put into cryotubes to be stored at -80°C. Spore solutions of *A. niger* strains were made by taking a fully colonized plate, to which 10 ml of ST was added and spores were collected by scraping them off from the plate using a spreader, after which the ST saturated with spores was sucked up and pressed through a syringe with cotton inside to block any mycelium or agar from going through. Spore solutions were stored at 4°C.

3.2 Plasmid isolation, construction and cloning

All primers used in this study are listed in supplementary table 1 (table S1) and all plasmids are listed in table S4. Plasmids were isolated from their respective *E.coli* strains using Midiprep or Miniprep kits (Margery-Nagel) according to the manufacturer's protocol. The first step in plasmid design was to pick a protospacer that would serve as the gRNA target for the Cas9 protein. By feeding CHOPCHOP (http://chopchop.cbu.uib.no/) with the locus of interest (up- and downstream regions of *suc1* (An08g11070, aspGD), *sucB* (An15g00320, aspGD) and *sucC* (An06g02420, aspGD)) a 23 base pair protospacer, including PAM sequence was given as output. Spacers were considered high-rated if the sequence was not self-complementary, had a GC content of >40% an efficiency of >65% and the cutting site would be near the gene of interest. Using this sequence, chimeric primers were designed that included the spacer sequence with either 18 bp of the TEF1 promoter or 23 bp of the TRACR terminator, resulting in the gRNA specific reversed and forward primer respectively. Using PCR, these primers were used to amplify two fragments using an existing gRNA plasmid as template, provided by Maarten Punt. A pre-existing forward primer, together with the gRNA specific reversed primer would result in a fragment consisting of the TEF1 promoter and the gRNA sequence of the gRNA primer. The gRNA specific forward primer, together with a pre-existing reversed primer resulted in a fragment consisting of the specific gRNA and the TRACR terminator. The samples of these two fragments were digested with DpnI

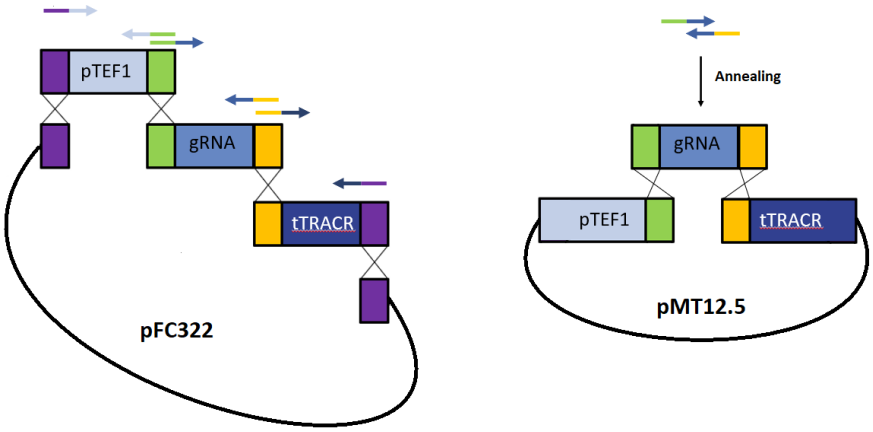

Figure 1. Different Gibson assembly methods. Arrows depict primers and the colors illustrate overlapping sequences.

(Thermo Fisher) to break down any remaining template. Both fragments were then mixed and ready to be ligated into a linearized backbone fig 1). Guide RNA (gRNA) plasmids were constructed using Gibson assembly. Oligos have been designed to serve as repair fragments in a transformation event to create the desired knockout strains (table S2). For a regular Gibson experiment, 3 µl of Gibson Assembly® Master Mix (NEB, #E2611L) was mixed with PacI (NEB) linearized pFC322, or Bts1 (NEB) linearized pMT12.5 as an alternative method. This new mixture was split for 2 reactions, to which one was added 0.5 µl of assembly DNA and the other 0.5 µl of MiliQ water as control. These tubes were incubated in a PCR machine at 50°C for 1 hour. In the meantime, competent *E.coli* stocks were thawed on ice 30 min before use. When the PCR machine was finished, the assembly and control mixtures were each added to a tube containing competent cells, gently spun down and incubated on ice for 1 hour. The cells were then heat shocked in a water bath at 42°C for 30 sec. 900 µl of SOC medium was added to the cells and incubated for at least 1 hour before plated on LB+amp plates and incubated overnight at 37°C. For the alternative Gibson assembly, the protocol of ligation is the same when using a pFC322 backbone, however the PCR of the assembly fragments is surpassed by taking the same designed primers, mixing them and putting them in a PCR machine at 95°C for 3 min, after which the temperature is slowly cooled to room temperature using a 4°C per 0.03 sec ramp setting. This way, the primers will anneal themselves, practically skipping one ligation step (fig 1). This can then be directly used as the assembly DNA and with linearized pMT12.5 as the backbone, the DNA will be placed at a location where the vector originally has a GFP, making it easy to exclude false positives when screening. Plasmids were screened using colony PCR with primer 1 and the unique reversed primer used for making the gRNA-terminator fragment for that particular plasmid. Correct plasmids produced a 267 bp band and were sent for sequencing (Macrogen Europe, Amsterdam, The Netherlands). Plasmid pMT9 was used as a GFP carrying vector and was created by digesting pMT8 with XbaI (NEB) and SacII (NEB). The pMT8 plasmid contains an inducible promoter that was not needed for this research and was therefore removed from the plasmid using this digestion. After ligating with T4 ligase (NEB), this results in a plasmid carrying a functional pyrG gene and a GFP cassette. pMT8 was kindly provided by Juan Pablo Moran Torres.

3.3 Protoplast release and transformation of *A. niger*

For *A. niger* transformation, donor strains were inoculated in 100 ml of liquid CM+GLU at a concentration of 10^6 spores/ ml for 18 hours in an incubator at 30°C with 200 rpm shaking. To make protoplasts, fresh mycelium was harvested using a Büchner funnel with a sterilized miracloth filter paper, washed with water and ST and the recovered mycelium was scraped off the filter and put in a falcon tube. An enzyme mixture consisting of 100 mg of BSA (A7030, Sigma), and 100mg of lysing enzymes from Trichoderma (L1412, Sigma) was dissolved in 20 ml of NMC (0.7M NaCl, 0.2M $CaCl_2$, 10mM MES, pH set to 5.6) and added to the mycelium. After incubating for 2 minutes for full hydration, the protoplast solution was vortexed and incubated at 37C at 200 rpm for 3 to 6 hours and each hour the protoplasts were counted. When there were at least 1*10^6 protoplasts, 20 ml of STC (1.33M sorbitol, 10mM Tris-Cl, 50mM $CaCl_2$) was added to the protoplast solution, carefully mixed and again harvested with a Büchner funnel with a sterilized filter paper. This time, the filtrate was collected and spun down for 10 min at 10C at 2000 rpm. Then the supernatant was discarded and the pellet was dissolved in 1ml of STC. This was now spun down for 5 min at 10C at 3000 rpm, this wash was repeated twice and in the meantime, MMS plates were poured. The donor DNA plasmids were freshly isolated before use and were assembled by taking 1000 – 4000 ng of DNA and if a co-transformation was set up, the co-transformation DNA was half the concentration of the donor DNA. In a single tube, STC was added to the donor DNA making it a total volume of 20 µl. This was added to the protoplasts and then 50 µl of PEG solution (25% PEG6000, 50mM $CaCl_2$, 10mM Tris-Cl) was slowly dropped on top. After that, 1 ml of PEG was slowly added and incubated at room temperature for 5 min. After incubation, 2 ml of STC was added and topped to 20 ml using liquid MMST with added resistance marker if needed. This was poured over the solidified MMS, until cool and incubated at 30C for 3-4 days. The procedure of screening positive clones is described in the next paragraph.

3.4 Making fluorescent *A. niger* strains

To increase the chances of success, several approaches in making the fluorescent strains were conducted. The first GFP strain was constructed by co-transforming the N400 wildtype (WT) strain with 4000 ng of pRB021 as donor DNA and 2000 ng of pAN7-1 as co-transformation DNA, making the strain N4G. The second GFP strain was created by taking strain AB4.1 where the pyrG gene has been made nonfunctional. This gene is responsible for uracil synthesis and transforming it with plasmid pMT9 and pPyrgG complements the pyrG gene and introduces GFP. The AB4.1 strain was gifted by Juan Pablo Moran Torres. Two red fluorescent strains were constructed by co-transforming with pRB069 together with pAN7-1 or pPhleo, resulting in strains N4RH and N4RP respectively. These plasmids were kindly provided by Dr. Robert-Jan Bleichrodt. Colonies were screened for their antibiotic resistance and green or red fluorescence. Positive clones were streaked on fresh PDA+hyg or PDA+Phleo plates and single colonies from this plate were restreaked twice on PDA plates to get rid of the antibiotic resistance containing plasmid and again screened for fluorescence, after which they were inoculated for stocking. Fluorescence on plates was observed using a Leica MZ 16FA microscope with GFP and RFP filters.

3.5 Genomic DNA isolation of *A. niger*.

To prepare genomic DNA for verification PCR after knockout transformations (see next paragraph), some mycelium from a suspected positive clone is transferred to an inoculation tube with 700 ul liquid CM+ GLU and grown overnight. This is then pelleted at 10.000 g for 3 minutes and washed twice with water. The pellet is then mechanically disrupted using a pipet tip and then

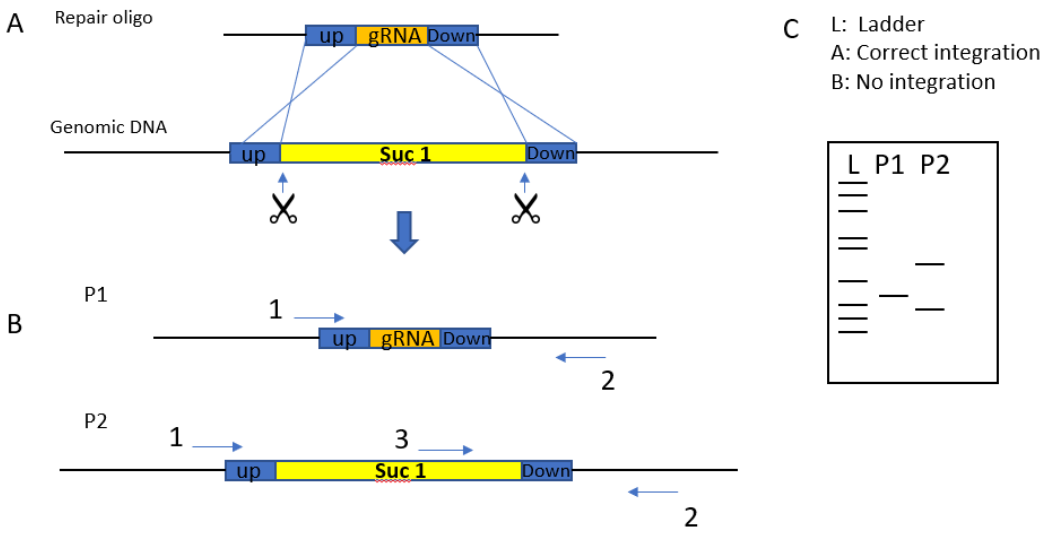

Figure 2. Construction and verification of mutants. A. An double crossover event by homologous ends of the repair oligo replaces the gene of interest. B. There's two possible outcomes of such a transformation; P1: correct integration, P2: no integration. C. Using primers 1-3, both possible outcomes give a different band pattern after gel electrophoresis.

resuspended in 500 ul lysis buffer (400 mM Tris-HCl pH 8.0, 60 mM EDTA pH 8.0, 150 mM NaCl, 1% sodium dodecyl sulfate). Now the sample is incubated at room temperature for 10 minutes. 150 ul of potassium buffer (60% 5M potassium acetate, 11,5% glacial acetic acid and 28,5% distilled water, pH 4.8) is added, vortexed and spun down at 10.000 g for 1 minute. The supernatant is transferred to a new tube and the spin is repeated after which the supernatant is transferred again to a new tube. To this tube, an equal volume of isopropanol is added and mixed by inversion. The tube is then spun down at 10.000 g for 2 minutes and the supernatant is discarded. The pellet is washed by adding 300 ul 70% ethanol and spinning down at 10.000 g for 1 minute. The ethanol is removed and the pellet dried after which it is resuspended in 50 µl TE buffer.

3.6 Making fluorescent sucrose knockouts

After the preparation of fluorescent WT strains, gRNA plasmids and repair oligo's, the sucrose mutants can be constructed by transforming as described in paragraph 3.3. To construct a single knockout strain, the two gRNA plasmids are added to the transformation mix. They will induce the CRIPSR protein to cut up- and downstream of the target gene. The repair oligo is added as donor DNA to replace the original gene. To confirm positive mutants, single colonies from the transformation plates are restreaked on PDA + hyg plates and after 2 to 3 days single colonies are taken again to be restreaked to PDA. Now, a single colony from each PDA plate is used to inoculate a pre-culture tube for genomic DNA isolation. After DNA isolation, a verification PCR is conducted using a set of 3 primers (fig. 2). The first primer binds in the upstream region of the gene, which is also present in the repair oligo. The second primer binds outside of the downstream region of the gene and the third primer binds to the original gene. This way, different band patterns are obtained for the two different outcomes; an successful integration of the repair oligo or the double cuts were repaired by the cells own repair mechanism. Using this approach, false positives can be excluded during screening.

3.7 Biomass determination of N4RH and ΔglaA

To determine the growth rates of strains N4RH and ΔglaA (Table s3), both strains were grown in 2 batches of 25 ml of MM+GLU and MM+ starch medium at a starting concentration of 10^6 spores/ ml with triplicates for each carbon source. The first batch was harvested after 16 hours and the second batch after 24 hours. Biomass was harvested by using a Büchner funnel, pouring the liquid medium onto a pre-dried, pre-weighted miracloth filter paper and applying vacuum. Filter papers with biomass were then put at 60C to dry for 24 hours after which the growth rate was determined by taking the difference in biomass between the two timepoints for each carbon source for each strain (fig. 3). The amount of biomass measured was averaged for all 16 hr and 24 hr samples per carbon source and from these averaged biomass numbers, the difference in biomass per hour was calculated. To determine mixed culture growth rates, this experiment was repeated but this time using inoculum ratios of 90/10, 50/50 and 10/90, N4RH to ΔglaA respectively, with an end concentration of 10^6 spores/ ml with biological triplicates for each carbon source in 2 batches, each for harvesting at the aforementioned time points.

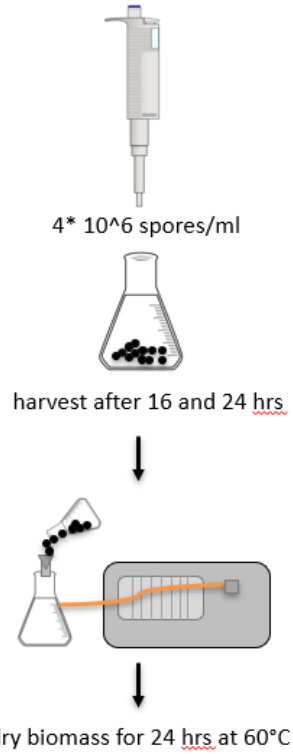

Figure 3. Setup of biomass experiment

3.8 Spore counts after co-culturing N4RH and ΔglaA

To test whether a glucoamylase mutant would grow together or maybe be outgrown by a WT strain, ΔglaA was co-cultured with N4RH by inoculating them mixed in flasks. Mixed cultures were inoculated in liquid MM+GLU and MM + starch with an end concentration of 10^6 spores/ml, both strains

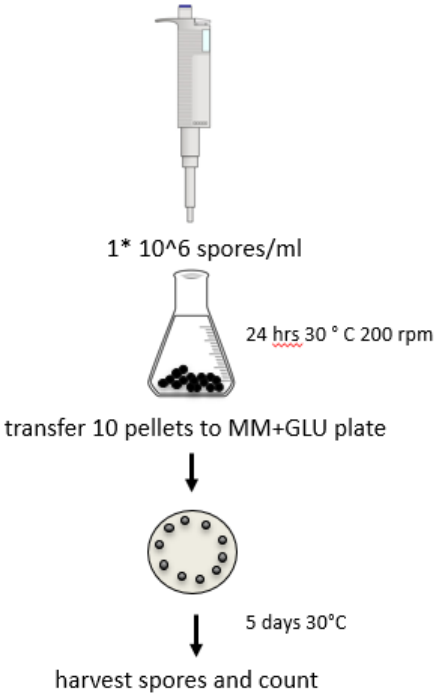

Figure 4. Setup of spore counts experiment

combined. The ratios were 90/10, 50/50 and 10/90 for the WT and ΔglaA respectively and were prepared in triplicates for each carbon source. After 24 hours of growth, 10 pellets of each flask were directly transferred to a MM+GLU agar plate to grow for 5 days. The spores were then harvested to make spore solutions of each plate. 2 ul of spore solution was diluted with 3 ul of ST and this was put on a slide to be put under the microscope (Zeiss Axioskop 2 Plus with RFP filter) to check which spores exhibited fluorescence (fig. 4). Pictures were taken with and without fluorescent filter using a SCMEX camera. Both pictures were edited in Microsoft PowerPoint by adding 8% transparency to the picture without the RFP filter. In the picture taken with RFP filter, transparency was set to 35% and by setting the saturation to 0, the spores turned grey. Now both pictures were layered on top of each other to create 1 complete picture showing regular spores in red and fluorescent spores in grey. By magnifying the view to 300x, this window frame was used to count 100 spores for each complete picture to determine the ratio of WT to ΔglaA spores.

3.9 Sporulation control experiment.

To test whether both N4RH and ΔglaA have similar spore production, both strains were individually inoculated on MM+GLU and MM+ starch plates with 10.000 spores in a single drop in biological triplicates (fig. 5). After 5 days the spores were harvested and counted using a haemocytometer.

3.10 Solid culture experiments of N4RH and ΔglaA

To support the findings of the liquid culture experiments, three growth experiments were conducted on MM+ GLU and MM+ starch plates (fig 6). In the first one, 10.000 spores for each strain from their respective spore solutions were inoculated on the opposite sides of the same plate. In the second plate experiment, 500.000 spores of each strain were homogeneously plated, each strain on one

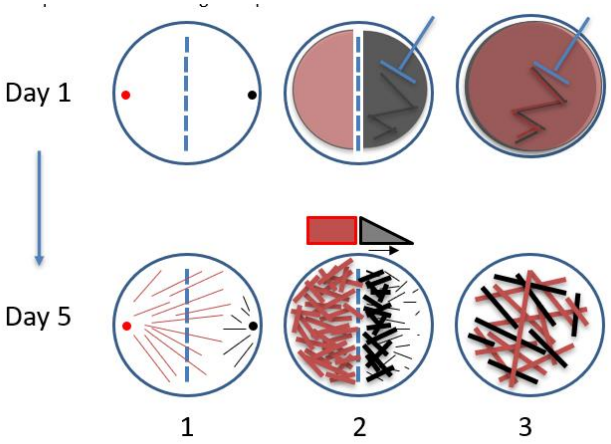

Figure 6. Setup of the different plate experiments. The spot of inoculated N4RH and its expected growth pattern is in red and that of ΔglaA in black.

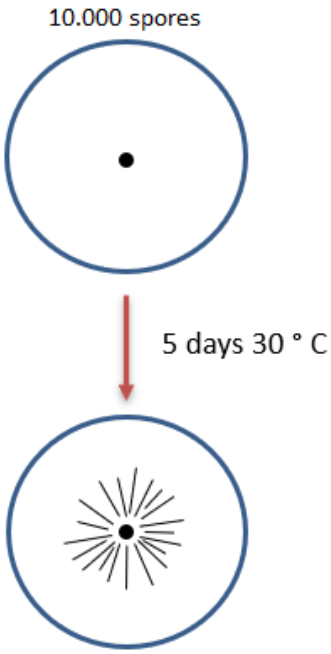

Figure 5. Setup of spore production experiment

Table 2. Ratios of N4RH to spores after harvest from the spore count experiment. Rows depict biological replicates for each inoculation ratio per carbon source.

Glucose			Starch		
90/10	50/50	10/90	90/10	50/50	10/90
46%	10%	1%	47%	3%	3%
64%	10%	1%	35%	4%	0%
50%	11%	2%	44%	7%	9%

half of the plate. In the final plate experiment a 50/50 mix of both strains was plated with an end concentration of 20.000 spores in total. All experiments were conducted in triplicates. For all plate experiments, growth was monitored for 5 days and spores were checked for fluorescence as described for the liquid culture experiment in the case of the 50/50 mix plates.

4. Results

4.1 Biomass determination

To determine the growth rate of each strain the differences in dry biomass after 16 and 24 hours of growth was determined by extracting the average biomass after 16 hrs from the average biomass after 24 divided by 8 hrs. This results in the maximum growth rate or µmax. In table 1, the calculated growth rates of the individual strains are shown.

Table 1. Growth rates as µmax for N4RH and ΔglaA on glucose and starch

µmax	N4RH	ΔglaA
Glucose	2.96	3.02
Starch	0.35	0.11

The data is based on one experiment. Growth on glucose resulted in similar growth rates with an µmax of 2.96 and 3.02 for the WT and mutant respectively. On starch, both strains grew significantly slower but the mutant grew slower than the WT. Here the WT had an µmax of 0.35 and the mutant an µmax of 0.11. This shows that the mutant has a growth deficiency on starch when grown in liquid culture.

An experiment determining the growth rates of the mixed cultures had been conducted but separately from the experiment in which the individual strains were done. Therefore, these results could not be compared and should be performed together in a new experiment to be conclusive.

4.2 Spore counts after co-culturing N4RH and ΔglaA

In the spore count experiment, the ratio of WT to mutant spores was determined after a transfer from liquid medium to plate. Plates were checked for fluorescence before harvesting spores and always showed bright fluorescence across the whole plate for both carbon sources. In table 2, the percentage of WT spores is depicted for co-cultures on glucose and starch respectively. None of the inoculation percentages are seen back in the ratio of WT to mutant spores after harvest. The highest percentage of WT spores that was found is 64% in a 90/10 inoculation ratio grown on glucose and the lowest percentage is 0% in a 10/90 ratio grown on starch.

4.3 Sporulation control experiment

As a control to the spore counts experiment, both strains were individually grown on both glucose and starch plates to produce spores that were harvested after 5 days and counted with a haemocytometer. The amount of spores was calculated by taking the average

from three replicates and taking the ratio of the total amount of mutant spores to the amount of WT spores.

. Table 3.Ratio of ΔglaA spores to WT

	Ratio of glaA spores to WT
Glucose	1.5
Starch	0.7

The ratio of spores of ΔglaA to WT is shown in table 3. Both strains show different spore production, where the mutant produces 1.5 times more spores on glucose compared to the WT but 0.7 times on starch.

4.4 Solid cultures of N4RH and ΔglaA

In the first solid culture experiment, both strains were inoculated on opposite sides to compare the growth speeds of both strains on solid culture. That resulted in the growth pattern as seen in figure 7. Both strains occupy a similar surface area and colony size on both carbon sources. In the second solid culture experiment, both strains were homogeneously spread, each on one side of the plate (fig. 8). On both carbon sources, the plates show full colonization and only the side where N4RH was inoculated there is fluorescence. In the final solid culture experiment, a 50/50 mix of both N4RH and ΔglaA was inoculated on MM+ GLU and MM+ starch plates (fig. 9). The plates show full colonization at the end of the experiment. However, sporulation seems more concentrated in some parts of the starch plates, compared to the glucose plates where sporulation occurs more or less homogeneously. Besides mycelial growth, fluorescence is also present across the whole plate for both carbon sources and was not affected by more or less concentrated sporulation.

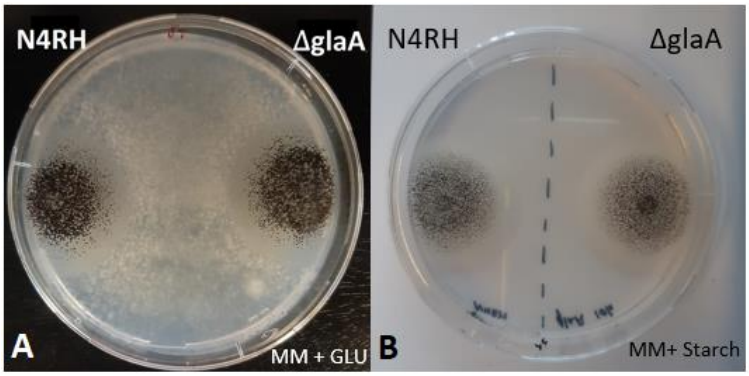

Figure 7. Solid culture experiment 1: equal inoculation of N4RH and ΔglaA on opposite sides of the plate, grown on minimal medium with glucose (A) and minimal medium with starch (B).

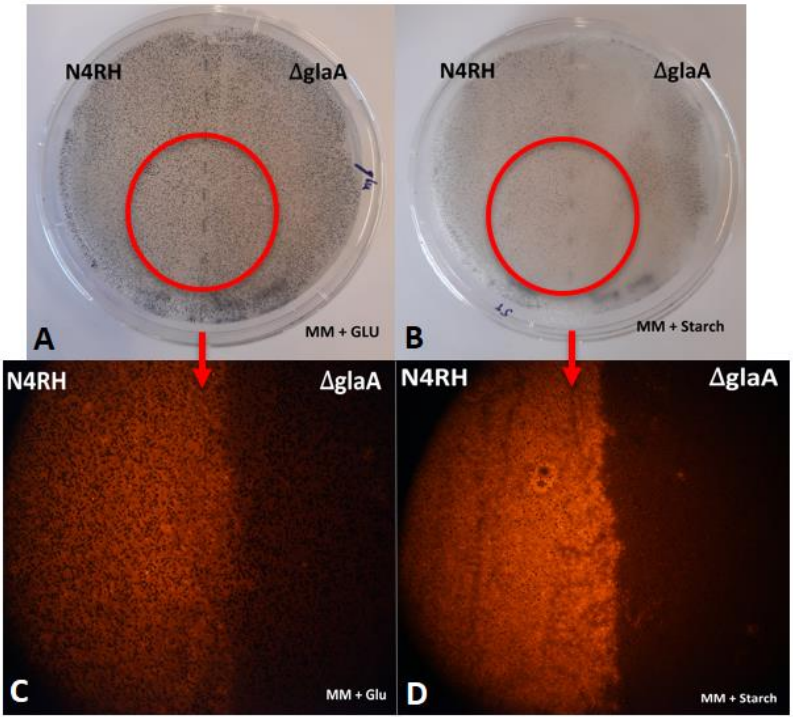

Figure 8. Solid culture experiment 2: inoculations of N4RH and ΔglaA each on one half of a minimal medium with glucose plate (A) and minimal medium with starch plate (B). Red circles are zoomed in sections of the glucose and starch plates that are showed underneath with fluorescent RFP filters (C, D).

5. Discussion

This research has focused on developing a sucrose knockout system that could be utilized to implement a standardized method for assessing cooperation or competition between a mutant and a wildtype. Even though the sucrose system has not been established due to limitations in time, the glucoamylase mutant was provided as an excellent alternative. Reflecting on the growth experiments, some finetuning is necessary in order to solidify the outcomes of the conducted experiments during this research. The biomass experiment had shown that it indeed has a growth deficiency on starch but shows different growth behaviors when comparing liquid and static cultures. However, it is important that all the different mixed cultures and single strains should be measured during a single experiment. Since spore solutions have slight variations each time, this would minimize the margin for variation during the experiment. For the spore counts experiment, an extra control should be added to the experiment where also 100% WT and 100% mutant spores are counted and checked for fluorescence. This to ensure that the WT did not lose its fluorescence over time, which could explain the low spore percentages that were obtained during the spore counts experiment. Furthermore, it was shown that the glucoamylase mutant grew comparable to the WT on plates. Since there are several glucoamylase enzymes present in *A. niger*. They are *gla*A, *gla*B an unnamed amyloglucosidase (An02g06950, aspGD) and an unnamed beta-glucanase (An02g00850, aspGD). Besides these genes, there are many other genes involved in starch degradation (Yuan, et al. 2008). The mutant still misses the most active glucoamylase, *gla*A, so it could be that the other secreted glucoamylases are not as highly expressed and because of the shaking, the cells cannot take up the diffusing sugars in liquid culture conditions. This could coincide with the fact that on solid agar

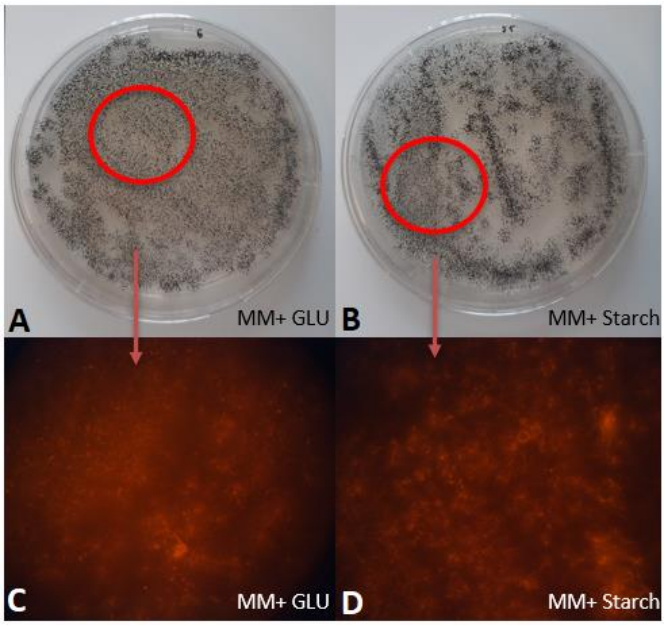

Figure 9. Solid culture experiment 3: inoculations of a 50/50 mix of N4RH and ΔglaA on a minimal medium with glucose plate (A) and a minimal medium with starch plate (B). Red circles are zoomed in sections of the glucose and starch plates that are showed underneath with fluorescent RFP filters (C, D).

plates, where the space for diffusion is also limited and the colonies are not shaken around, that these genes are sufficiently expressed to support growth for a growth rate similar to the WT. To rule out residual growth, knockouts should be made of the remaining glucoamylase genes, as well as introducing a fluorescent marker. The expected phenotype here being that it would not grow on starch whatsoever and fluorescent microscopy would make it easier to confirm this. This would make the mutant more dependent on the WT when mixed on starch. This principle would be the same for the sucrose system. However, if the mutant is indeed a cheater then it is worth investigating this further. A follow up experiment could be to use the spores harvested from the spore counts experiment and to use inoculate another liquid culture. It would be interesting to see whether the WT would be outcompeted by the mutant over the selection of reproduction. This would result in an even slower growth rate, since this new cheater cell lineage would be selecting solely

for efficient reproduction instead of its investments in somatic functions which hinders the fitness of the population (Bastiaans, Debets and Aanen 2016). Still to the best of my knowledge, co-culturing *A. niger* with a mutant of itself for the purpose of testing growth behavior has not been reported and thus worth investigating from a fundamental perspective. Hopefully the sucrose system, once in completion, could support the findings of this research and unravel other phenomena around heterogeneity in *A. niger*. Sucrose is an excellent choice for such a setup since as has been shown in S. cerevisiae, where the invertase gene is also secreted into the medium, that it requires cooperation from multiple cells to grow on this sugar. This is because a single cell could never capture the monosaccharides because the molecules diffuse. Therefore in a bigger, cooperative colony, the neighboring cells are able to pick up the diffusing sugars and thus allow growth (Koschwanez, Foster, and Murray 2011). Such an experiment with single spores from an *A. niger* WT and a sucrose mutant would be an interesting setup to verify if indeed WT secretion supports growth of the mutant. However, such experiments still only suggest a certain interaction given that it is judged by macro level observations. To uncover what mechanisms are responsible for these interactions, molecular tools should be applied. It is possible that *A. niger* contains a mechanism for recognizing other fungi that are compatible for cooperation. Even though *A. niger* is regarded an asexual species, some mating-type genes have been reported as well as het-genes, that are responsible for the attraction or rejection of partners during the parasexual cyclus (Pál et al. 2007). This shows that there is a complex molecular mechanism at play that perhaps also influences *A. nigers* position towards cooperation.

6. Conclusion

This research shows that a standardized method can be set up for assessing interactions in co-cultures. The sucrose-system could validate itself as a valuable proof of concept once applied to this setup. This can ultimately bridge the knowledge gap we currently have about heterogeneity and cooperative interactions in *A. niger*.

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Primer	Sequence	Description
suc1UgR.Rv	CAGTTGTTTTCCCTTCCCGAGACGAGCTTACTCGTTTCG	Reverse primer to amplify pTET1 with a 3' gRNA tear for the suc1 upstream target site
suc1UgR.Fwd	TCGGGAAGGGAAAACAACTG GTTTTAGAGCTAGAAATAGCAAG	Forward primer to amplify tTRACT with a 5' gRNA tear for the suc1 upstream target site
suc1DgR.Rv	TCGGGAAGGGAAAACAACTGGACGAGCTTACTCGTTTCG	Reverse primer to amplify pTET1 with a 3' gRNA tear for the suc1 downstream target site
suc1DgR.Fwd	CAGTTGTTTTCCCTTCCCGAGTTTTAGAGCTAGAAATAGCAAG	Forward primer to amplify tTRACT with a 5' gRNA tear for the suc1 downstream target site
pTE1_Fwd.q	GTTTCCGCTGAGGGTTTAATACTCCGCCGAACGTACTG	Forward primer to amplify pTET1 with a 5' 20 bp tear for the proto-final CRSPR backbone
pTE1_Rev.q	CTGTCTCGGCTGAGGTCTTAAAAAGCAAAAAAGGAAGGTACAAAAAAGC	Reverse primer to amplify tTRACR with 3' a 20 bp tear for the proto-final CRSPR backbone
sucBUgR.RV	CGAGCTTTATCGGTGGGGGTGACGAGCTTACTCGTTTCG	Reverse primer to amplify pTET1 with a 3' gRNA tear for the sucB upstream target site
sucBUgR.Fwd	ACCCCCACCGATAAAGCTCGGTTTTAGAGCTAGAAATAGCAAG	Forward primer to amplify tTRACR with a 5' gRNA tear for the sucB upstream target site
sucBDgR.Rv	TTGTCACCTTTTACGGGGTAGACGAGCTTACTCGTTTCG	Reverse primer to amplify pTET1 with a 3' gRNA tear for the sucB downstream target site
sucBDgR.Fwd	TACCCCGTAAAAGGTGACAAGTTTTAGAGCTAGAAATAGCAAG	Forward primer to amplify tTRACR with a 5' gRNA tear for the sucB downstream target site
SucCUpgRNA.Rev	CCATGGCATCAAGGGACAAGGACGAGCTTACTCGTTTCG	Reverse primer to amplify pTEF1 with a 3' gRNA tear for the sucC upstream target site
SucCUpgRNA. Fwd	CTTGTCCCTTGATGCCATGGGTTTTAGAGCTAGAAATAGCAAG	Forward primer to amplify tTRACR with a 5' gRNA tear for the sucC upstream target site
SucCDngRNA. Rev	TCCGAATAGCTGCGCGGCACGACGAGCTTACTCGTTTCG	Reverse primer to amplify pTET1 with a 3' gRNA tear for the sucC downstream target site
SucCDngRNA. Fwd	GTGCCGCGCAGCTATTCGGAGTTTTAGAGCTAGAAATAGCAAG	Forward primer to amplify tTRACR with a 5' gRNA tear for the sucC downstream target site
Cas_Common_verify_Fw	ACTCCGCCGAACGTACTGGTA	Common forward primer for Cas9 verification
Cas_Common_verify_Rv	GGCTTAACTTGGGCGGTGAT	Common reverse primer for Cas9 verification
gDNAsuc1.Fw	CGAGCAAGCTAGATTCCACAC	Forward primer to amplify the oligo sequence that replaced suc1 during an transformation event
gDNAsuc1.Rv	GGAGCTGGATGAAGTGCTTT	Reversed primer to amplify the oligo sequence that replaced suc1 during an transformation event
gDNAsucC.Fw	GCCGTCAGCTCAATTTTAGACAG	Forward primer to amplify the oligo sequence that replaced sucC during an transformation event
gDNAsucC.Rv	TTCCATAGGCTCTGCGCTT	Reversed primer to amplify the oligo sequence that replaced sucC during an transformation event
kusA_Fwd	CTC CGG GTT GAT CTT GTC C	Forward primer to amplify the A.niger kusA gene
kusA_Rv	CAT CGC ACC AAC CAG CAC G	Reverse primer to amplify the A.niger kusA gene

Table S1. Primers used in this study

Oligo	Description
suc1Repair	repair oligo to replace suc1 in a double cross over event
sucBRepair	repair oligo to replace sucB in a double cross over event
sucCRepair	repair oligo to replace sucC in a double cross over event
kusA repair	repair oligo to remove the gRNA sequence from kusA and inserting a stopcodon in the middle
	Sequence
suc1Repair	TAGATTCCACACCAGGTAACTTCAGTCAAGCCAACAGAACCTCGGTAGGGATAACAGGGTAATGGGGGTGTTTTCCCTTCCCGATACTTTACTGAACTGATACACGATGAAGA
sucBRepair	CTGCATTGGATTTTGCAGCGGCTGCCAATTCACTCCCGGACCTCGTAGGGATAACAGGGTAATGGGGGAAGGGTTTTGAATTCGCCGACGATGACGTAAATGAGGGTGAGTGT
sucCRepair	GTTTAACTATCAAAACATAAAAAGGCAACCCTTGTCCCTTGATGCTAGGGATAACAGGGTAATGGGGGGGAAGGAGGTACAGACATGTGCTGTATGCTACCCAGCCCCAACAG
kusA repair	TTTTTCGAACATGCCTTTAGAACTTGGCCCAGACTTCAGAATTTCGGTATTgaAGGCAAGCGCCCGCTAGAAACTCCTTCATCTGGCTGAACGGCGAGAAGCC

Table S2. Oligos designed for this study

Strain	Parental strain	Features	Reference
N400	NRRL3	Standardized lab strain	Bos et al., 1988
N4RH	N400	RFP, hyg	this study
N4RP	N400	RFP, phleo	this study
N4G	N400	GFP, hyg	this study
AB4.1	N402	pyrG-	van Hartingsveldt et al., 1987
AB4.1G	AB4.1	GFP, pyrG-	this study

Table S3. Strains used in this study

Plasmids	Features	Reference
psuc1Up.g	AmpR, HygR, Cas9, TEF1, TRACR RNA terminator, upstream gRNA to target suc1	this study
psuc1Dn.g	AmpR, HygR, Cas9, TEF1, TRACR RNA terminator, downstream gRNA to target suc1	this study
pRB069	AmpR, RFP	Bleichrodt, 2012
pRB021	AmpR, GFP	Bleichrodt, 2012
pAN7.1	AmpR, HygR	Universal transformation vector
psucBUp.g	AmpR, HygR, Cas9, TEF1, TRACR RNA terminator, upstream gRNA to target sucB	this study
psucBDn.g	AmpR, HygR, Cas9, TEF1, TRACR RNA terminator, downstream gRNA to target sucB	this study
psucCUp.g	AmpR, HygR, Cas9, TEF1, TRACR RNA terminator, upstream gRNA to target sucC	this study
psucCDn.g	AmpR, HygR, Cas9, TEF1, TRACR RNA terminator, downstream gRNA to target sucC	this study
kusA.gRNA	AmpR, Hyg, gRNA to target kusA	provided by Juan Pablo Moran Torres
pMT8	AmpR, pyrG, TetOn, GFP	provided by Juan Pablo Moran Torres
pMT9	AmpR, pyrG, GFP	this Study
pyrG locus	AmpR, hyg, gRNA to target pyrG	provided by Juan Pablo Moran Torres

Table S4. Plasmid used in this study