Improving glucoamylase activity of black aspergilli using experimental evolution

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Abstract

Filamentous fungi are of great industrial value due to their ability to excrete large amounts of enzymes. In this study we investigate the possibility of improving starch degrading glucoamylase by experimental evolution. By allowing 80 parallel lines of *A. niger* to acquire mutations over 240 generations we were able to select for mutants with higher fitness on starch medium. In addition, we screened 30 natural isolates from a worldwide collection on starch use efficiency, providing insights into new starting material for strain improvement.

The black Aspergilli, with model organism *Aspergillus niger* in particular (Kwon et al., 2012; Meyer et al., 2011), are filamentous fungi with a long history of use as industrial enzyme producers. The capacity to excrete extracellular enzymes in large amounts, ranging up to 20 grams per liter of medium (Finkelstein, 1987), combined with the ability to express a vast diversity in primary and secondary metabolites such as proteins and organic acids makes this fungus a useful cell factory in many branches of industry. Its tolerance to diverse growing conditions raises possibilities for culturing in broad ranges of pH, salinity and temperature. In addition to this, *A. niger* is able to degrade low-cost and abundant substrates such as starch, cellulose and pectin.

The most abundant of the extracellular enzymes secreted by *A. niger* is glucoamylase (E.C. 3.2.1.3, glucan 1,4- α-glucosidase, γ-amylase) which can make up more than 50% of excretome in liquid culture (Lu et al., 2010). Glucoamylase is one of the most commonly used enzymes in the food industry (Reilly, 1999) as it is useful in conjunction with α-amylase by its ability to almost fully convert starch into free glucose by hydrolyzing both α-1,4 and α-1,6 linkages (Kumar and Satyanarayana, 2009).

Interest in strain improvement for glucoamylase production has historically been high and many methods have been successfully used to increase glucoamylase production. In the past strategies such as random mutagenesis (Suresh et al., 1999) and directed evolution (Wang et al., 2006) have been used to improve glucoamylase overall yield or specific desirable properties such as increased thermostability. More recently *A. niger* has become a model species for many industrially relevant fungi (Kwon et al., 2012) as the public availability of ten genomes of the most important Aspergilli since 2010 have opened up new possibilities for research.

Information is a crucial bottleneck in directed strain improvement. Despite the recent advances that were made, the most commonly used methods share a common dependence on detailed information about the processes that need to be improved: recombination requires genetic markers, whereas the effect of inserting multiple gene

copies via genetic engineering depends on the specific location of insertion and gene copy number. Directed evolution, although very effective in selecting single beneficial mutations in a gene is restricted by the effects of epistasis (Suresh et al., 1999; Wang et al., 2006) and its application is usually limited to the protein coding part of a single gene (Dalby, 2003).

Recent transcriptomic and metabolomic research (Lu et al., 2010) has provided novel insights into glucoamylase metabolism and can reveal potential targets for directed strain improvement. Furthermore, selection systems based on markers such as hph, ble, oliC3 (antibiotic resistance); pyrG, pyrE, argB, adeA, adeB, niaD, trpC, sC (auxotrophic); and amdS, ptrA (nutritional) have been developed (Jin et al., 2004; Meyer et al., 2011), which in combination with recent versatile vectors offer good and flexible control for genetic manipulation of *A. niger* strains. These novel genetic engineering strategies for expression of heterologous proteins by *A. niger* have been recently reviewed (Fleißner and Dersch, 2010).

However, all methods mentioned above have the drawback of utilizing only a small part of the producing organism's potential: they focus on a specific factor that is limiting protein production which can be resolved using the strengths of a specific technique. Once this bottleneck is removed there will be a different step in protein biosynthesis that is rate-limiting. Apart from the focus on specific bottlenecks, this approach to optimization is limited by the techniques available and the imagination of the researcher that is using them.

Here we propose a novel, more holistic approach to increase the enzyme production and activity of glucoamylase by black Aspergilli, especially *A. niger,* using experimental evolution. Experimental evolution has opened up a new way of studying evolution as the details of many ecological and evolutionary questions were previously only resolvable trough theoretical analysis or comparative approaches, making it difficult to test the assumptions that are required to model evolution. In laboratory based experimental evolution the fast generation time and small size of microorganisms is put to use in studying the dynamics of evolution. In the lab, relatively large populations of microorganisms can be maintained under controlled circumstances that allow detailed monitoring of both phenotypes and mutations while assessing their fitness effects on a timescale spanning many generations. In addition, the lab provides the possibility to store viable microorganisms at very low temperatures, essentially creating a frozen fossil record that can be used to test the reproducibility of evolution by re-evolving from a previous generation, almost as if we could rewind the proverbial tape of life. This allows researchers to directly compete an evolved organism against its ancestor to investigate whether competitive fitness is increased in the descendants of this ancestor.

In our approach the whole organism is allowed to evolve under conditions that favor specific properties i.e. high glucoamylase activity, desired by the experimenters. Now the focus is not on any specific target gene or technique, but the system as a whole is allowed to adapt to a novel environment.

By growing *A. niger* on a substrate that confers a fitness benefit to increased glucoamylase activity we can select for mutations that increase both yield and activity of the enzyme without the need to focus specific details of the fungal biochemistry or genetics.

Specifically, our study aims to test whether it is possible to select strains that are specifically adapted to using a complex carbon source such as starch by using experimental evolution. We selected 8 different strains of black aspergilli, and allowed 10 replicated lineages of each strain to evolve on solid medium with soluble starch as the sole carbon source for 240 generations, by periodically transferring replicate evolving lineages to fresh medium. In this way imposing conditions where spontaneous mutations that increase γ-amylase activity are favored by selection. After 240 generations, we assayed changes in fitness in various conditions, specifically assaying whether the increase in fitness was due to a specific adaptation to growing on starch medium, rather than general adaptation to laboratory conditions.

Materials and Methods

Strains used

The fungal strain strains were assigned a letter from A to H. As is shown in table 1. A. niger 402 is a commonly used lab strain while A. niger 402 B1 is a high glaA copy-number strain A. niger 402 Δgla was obtained through Peter Punt (University of Leiden, The Netherlands). All other strains were obtained from the collection of the Laboratory of Genetics, Wageningen University

Table 1: strains used in the selection experiment

Strain	Origin	Species	Reference
ind 2.1.5	Natural (A)	A. niger	
F 1.2.2	Natural (B)	A. tubingensis	
N 402	Lab (C)	A. niger	
N 402B1	Lab high (D)	A. niger	(Verdoes et al., 1994)
N917 // B13	Diploid (E)	A. niger	(Verdoes et al., 1994)
N915 // B3	Diploid (F)	A. niger	(Verdoes et al., 1994)
ind 1.8A.10	Hypermutator (G)	A. niger	

Media

Minimal Medium (MM) is $NaNO_3$ 6g/L^{-1}; KH_2PO_4 1,5g/L^{-1}; $MgSO_4 \cdot 7H_2O$ 0.5 g/L^{-1}; KCl 0.5 g/L^{-1} and 1 mg of trace elements $FeSO_4$, $ZnSO_4$, $MnCl_2$, and $CuSO_4$ set to pH 5.8 15 g/L^{-1} electrophoresis grade agarose (Invitrogen, Cat No: 15510-027) was used with Millipore Milli-Q water.

Carbon sources were 15 g/L^{-1} or 1.5 g/L^{-1} General purpose grade soluble starch Fisher Scientific CAS No. 9005-25-8), Glucose15 g/L^{-1} and maltose 15 g/L^{-1}

All glassware for medium preparation was rinsed with 0.1M HCL in MQ-water to remove traces of Ca^{2+}. All cultures were incubated at 30°C.

Selection experiments

We initiated 10 replicate populations from 8 black aspergillus strains by placing 5 µl of a dense spore suspension (min. 6000 spores) at the front of a cell culture flask (Greiner 50 ml, 25 cm^2CELLSTAR® sterile Cell Culture Flasks with filter caps, Cat-No. 670195) containing 10 ml of solid Minimal Medium (MM) with 1.5 % soluble starch as carbon source. Every 7 days the flasks were inundated with 5 ml of saline (0.8%)–Tween80 (0.005%) solution to extricate the fungal spores and a random 5 µl sample of spore solution (containing $6x10^3$ - $6x10^4$ spores) was transferred to fresh medium in a new flask. This random sampling of spores has proven to be effective before (Schoustra et al., 2006).

We propagated these 10 x 8 = 80 replicate lineages by serial transfer for a total of ~240 generations (~ 40 generations per 7 d) As glucose is the end product of starch degradation by glucoamylase, we grew all strains on glucose medium for one week prior to the selection experiment in order to prime the strains metabolism for higher glucose use.

Before harvesting spores, the Mycelial Growth Rate (MGR) was measured as a commonly used and highly repeatable (Pringle and Taylor, 2002; Schoustra and Punzalan, 2012; Visser et al., 1997) proxy for fitness. An aliquot of the transferred sample was stored in glycerol-neopeptone at -80°C.

After 6 transfers we inoculated 80 flasks containing standard medium and 80 containing low starch medium (10% of the standard starch concentration) in order to assay the effects of increased selection pressure by lower availability of the carbon source. These flasks were grown for three weeks without transferring. MGR was measured with weekly intervals and after three weeks we quantified spore biomass as described in (Schoustra and Punzalan, 2012) as additional component of fitness.

Selection on increased glucoamylase activity

Although glucoamylase is the most abundant protein in the excretome of *A. niger* growing on starch, α-amylase (a Ca^{2+} dependent metalloenzyme) is also present and is likely to be under the same selective pressure as glucoamylase. To minimize selection on α-amylase activity we used a solid medium that was free of Ca^{2+} to disrupt the catalytic activity of α-amylase, allowing for selection on glucoamylase.

Specific adaptations to starch

To test for specific adaptation to degradation of soluble starch we quantified spore production via image analysis on MM with either glucose, maltose or soluble starch as carbon source. This provided a high throughput method with good discrimination between strains which allowed for the screening of a selection of the evolved lines and natural isolates.

Measures of fitness

MGR on different carbon sources was measured in triplicate by placing 5 µl of a dense spore suspension in the centre of a Petri dish with solid MM. After 7 d of incubation, the diameter of the resulting fungal colony was measured in two perpendicular directions as described in (Schoustra et al., 2009). MGR during experimental evolution was measured for each replicate by assessing linear growth over the longitudinal axis of the cell culture flasks.

The spore biomass (BM) was estimated by image analysis using ImageJ version 1.48 for Windows. Images were taken using a Nikon D80 camera with Nikon 60mm f2.8 AFD micro objective at F8, ISO 100. Images were cropped, color thresholded and measured via the batch macro function (see Supplementary Data figures S1 and S2).

For the selection experiment we determined fitness (*w*) of genotypes as the MGR after 7 d of incubation on MM starch. Relative fitness was calculated as *w* evolved/*w* ancestor.

Dutch natural isolates

In addition to the isolates that were obtained from the collection of the Laboratory of Genetics, a number of new strains were isolated. For each natural isolate, 1 gram of soil was inoculated in Complete Medium + 20% tannic acid as described previously (Van Diepeningen et al., 2004)

Results and discussion

This study aims to test the possibility to select for increased glucoamylase production by fungal strains via experimental evolution by using starch as a carbon source. After 240 generations of evolution we tested for specific adaptations to starch by assaying BM and MGR as proxy for fitness on different solid media.

Selective effect of the medium

The assumption that Ca^{2+} depletion disrupts the catalytic activity of α-amylase was tested with *A. niger* 402 Δ*glaA*. Figure 1 a and b show the effect of Ca^{2+} depletion of the medium on growth three days post inoculation. The negative control *A. niger* 402 Δ*glaA*, lacking glucoamylase shows almost no growth on Ca^{2+} depleted medium. Addition of Ca^{2+} can rescue the phenotype by allowing for starch degradation through activity of α-amylase.

Figure 2 a and b show the selective strength of the medium: the high *glaA* copy-number strain *A. niger* 402 B1 grows faster and more dense then *A. niger* 402 which is physiologically identical except for the *glaA* copy number. Faster and denser growth provides competitive fitness so that in a mixture of strains the recombinant N402 B1 would outcompete N402.

Diversity in natural isolates

The type of mutations that are available to selection will have a strong influence on the outcome of selection in this setup. Furthermore, differences in starting genotype can provide a diverse range of adaptive responses to selection. Therefore we investigated the differences in carbon source utilization of natural isolates synchronously with the evolved lines.

Here we found diversity in both the total level of spore production (figure 3a and 3b) and MGR (data not shown) of natural isolates. Isolate WA3 shows an increase in BM of 4.5x on MM starch when compared to B2.11 (figure 3b).

The large diversity in total spore number is accompanied by diversity in the reaction norms for carbon source: figure 3b shows differences in spore production on MM glucose for strains that were isolated less than 500 meters apart (WA1 vs WA2 and AR1 vs AR2). The natural isolates that were used for this selection experiment were not primarily adapted to starch before the experiment, therefore screening natural diversity for strains performing better on starch can provide novel starting points for strain improvement.

Selection experiment

The box-plots in figure 4 show the relative fitness increase in MGR of the evolved genotypes per line. The mean fitness increase over all eight lines is 1.1636, indicating an increase in fitness of 16 percent over the ancestral genotype which is statistically significant (t-test; t_{80}= 7,808; P < 0.005).

Mycelial growth rates of ancestral and evolved genotypes are shown in table 2 and figure 5. MGR differences were tested for significance using a one-sided t-test assuming unequal variances with Holm-Bonferroni correction on *p*-value.

Table 2: t-test (one-side) between means of MGR in mm for eight lines. *ns*= not significant with Holm-Bonferroni.

Line	Ancestor (MGR)	Evolved (MGR)	*p*-value
Natural (A)	20	21,6	0,014621
Natural (B)	20,6	24,2	0,007263
Lab (C)	15,9	19	0,000174
Lab high (D)	19,4	20,2	0,028 *ns*
Diploid (E)	18,6	21	7,31E-05
Diploid (F)	18	23	3,5E-07
Hypermutator (G)	18,5	25,9	5,3E-12
Hypermutator (H)	19,8	19,1	0,0651 *ns*

The hypermutator (G) genotype gives the strongest response to selection in this experiment, while hypermutator (H) shows a slightly (*ns*) negative response. The Lab high (D) strain, carrying more *gla*A copies, has a significantly higher MGR then Lab strain (C), however its response to selection is not

significant. This absence of a response to selection for Lab high (D) can be due to a limited number of beneficial mutations that were available to selection due to physiological constraints by the high *glaA* copy number.

When increasing selection pressure by reducing the amount of available energy by 90% we observed a significant negative response in BM over all strains: mean BM = 30.06 mg, SD= 3.4 mg on 1,5% starch; mean BM = 24.66, SD= 1.6 mg on 0.15% starch. The response in MGR was not significant: mean MGR = 63,11 mm, SD= 11,26 mm on 1,5% starch; 60,30 mm, SD = 12,38 on 0.15% starch. This indicates a potential response to low energy availability by decreasing spore production. Therefore, when testing for specific adaptation to starch on media with different carbon sources, we measured both MGR and BM as proxy for fitness.

Specific adaptation to starch

Figures 6, 7 and 8 show the evolutionary response in BM and MGR of strains on MM with either starch, maltose or glucose as the single carbon source.
The evolved lines of natural isolate A3 (figure 6a) have a relative fitness of 1,22 (BM) and 1.07 (MGR) using starch as carbon source while on glucose the relative fitness decreases to 0.94 (BM) and 0.88 (MGR). This suggests a specific response to starch utilization: adaptation to the medium itself or to more efficient energy use in general would lead to an increase in relative fitness in glucose use as well.

Lab strain (C) (figure 7a and b) shows a similar pattern were the relative fitness increase on starch is higher than on glucose. Strain C4 shows a higher relative fitness in BM on maltose (1,4) than on starch (1,2). As g*laA* production is upregulated by maltose and maltose is effectively degraded by glucoamylase, adaptation to starch degradation by increased glucoamylase activity is expected to increase maltose degradation simultaneously. The exact mechanism responsible for increased relative fitness here is unknown. However, the diversity in adaptive patterns seen in figures 7a and 7b suggests the involvement of multiple adaptive routes.

This idea is strengthened by the Hypermutator strain (G10) were the response in BM is significantly higher than in MGR: high spore production will increase reproductive fitness and considering the nature of our experimental setup, a high spore production can be favored by selection independent of increases in MGR (see figure 8a and 8b).

Fitness proxies

Spore production (BM) is used here as a proxy for biomass and fitness. It offers good discrimination between strains and it can reveal cases were the BM/MGR ratio is distorted, see figure 8a and 8b. Despite these benefits, acquiring BM data is laborious and error-prone. In addition to this, the BM data are highly skewed (see figure S5 and S6) making data transformation necessary before statistical analysis.
MGR data are less laborious to acquire and approximately normally distributed (see figure S7). The covariance for BM/MGR is 1.48 for the natural isolates, making MGR a more convenient proxy for fitness.

Ultimately, the true test of increased fitness over an ancestral genotype is a head-to-head competition on a single plate. However, these experiments are notoriously error prone. Consequently, the number of replicates needed for proper statistical analysis makes this method laborious and less preferable. Therefore, we refrained from performing these experiments on a large scale. MGR has been used instead as it has been

shown to correlate with the competitive ability of strains in head-to-head competitions (Schoustra et al., 2009).

In summary, the results presented here show the possibility of improved growth on starch as carbon source by specific adaptation of the filamentous fungus *A. niger* by means of experimental evolution. In addition we show that there is diversity among natural isolates in their growth on starch which can provide insights into novel starting strains for improvement of glucoamylase activity by either experimental evolution or traditional approaches.

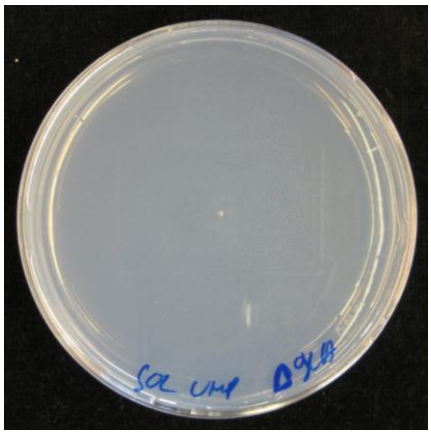

Figure 1a *ΔglaA* on soluble starch

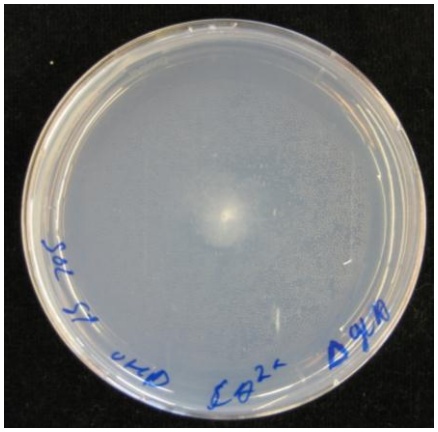

Figure 1b Δ*glaA* on soluble starch supplemented with CaCL, which rescues the phenotype.

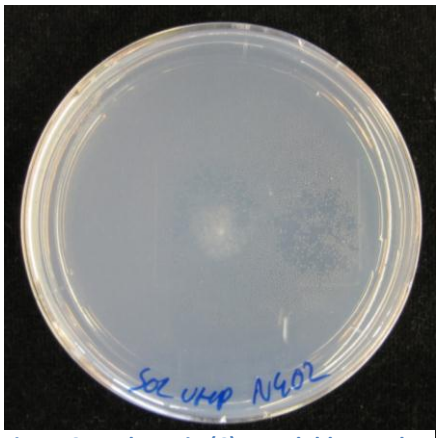

Figure 2a: Lab strain (C) on soluble starch medium after three days of growth.

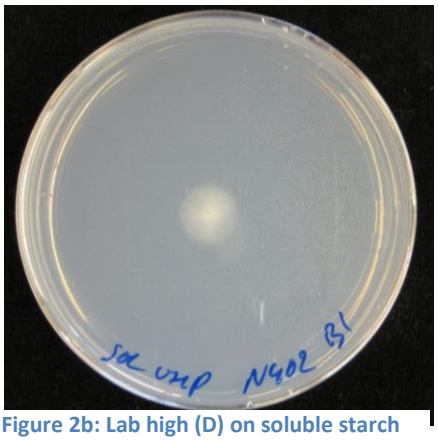

Figure 2b: Lab high (D) on soluble starch after three days of growth, the mycelium is much denser when compared to figure 2a

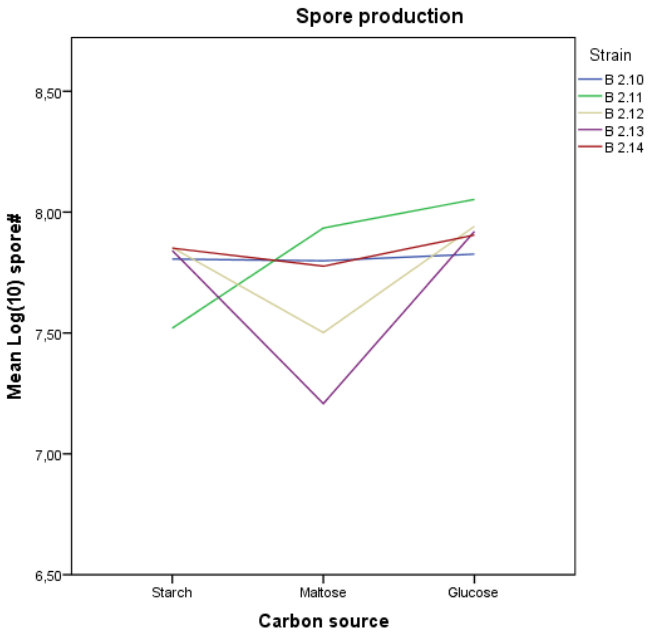

Figure 3a: spore production of natural isolates. Strains were isolated in Brazil and obtained from the Laboratory of Genetics in Wageningen, The Netherlands

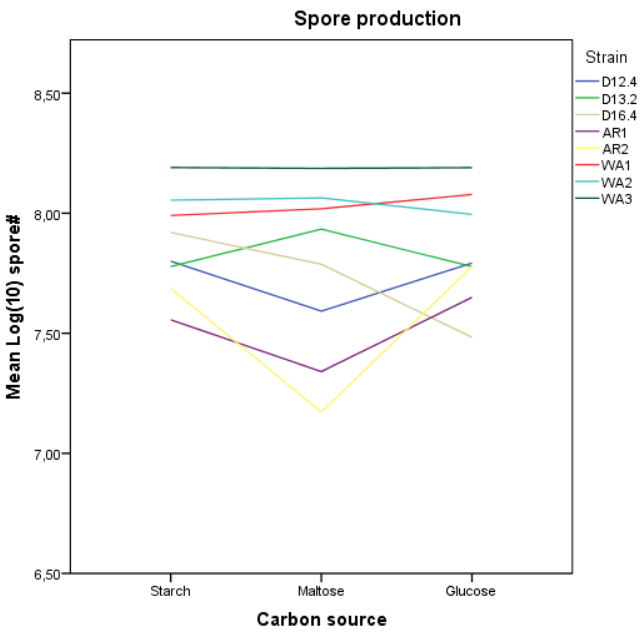

Figure 3b: spore production of natural isolates. Strains were isolated from Delft (D), Arnhem (AR) and Wageningen (WA)

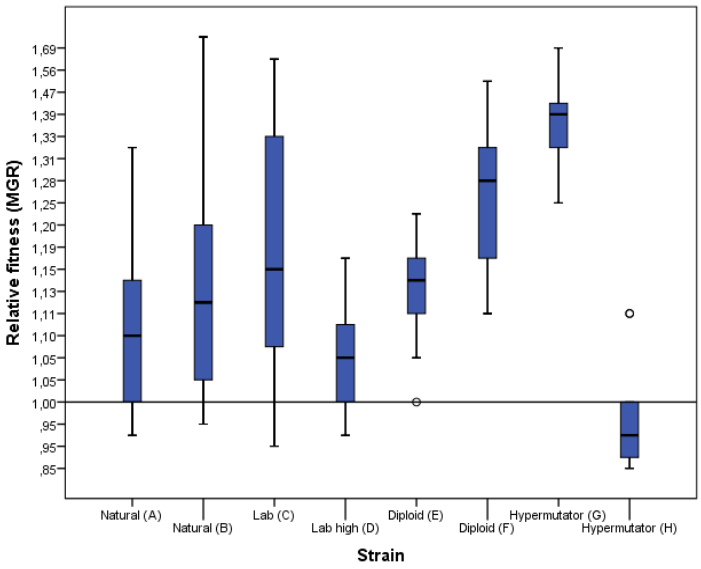

Figure 4: Box plots of relative fitness by MGR, the box represents quartile one and three, the black bar is the median, whiskers are min and max values.

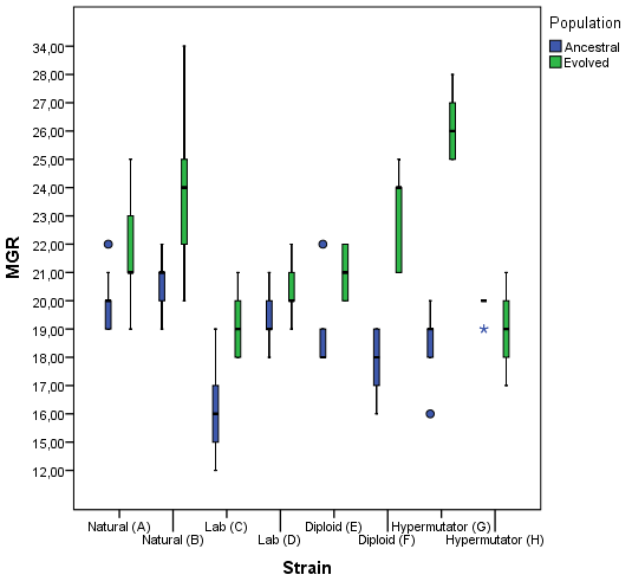

Figure 5: Box plots of MGR data. The box represents quartile one and three, the black bar is the median, whiskers are min and max values.

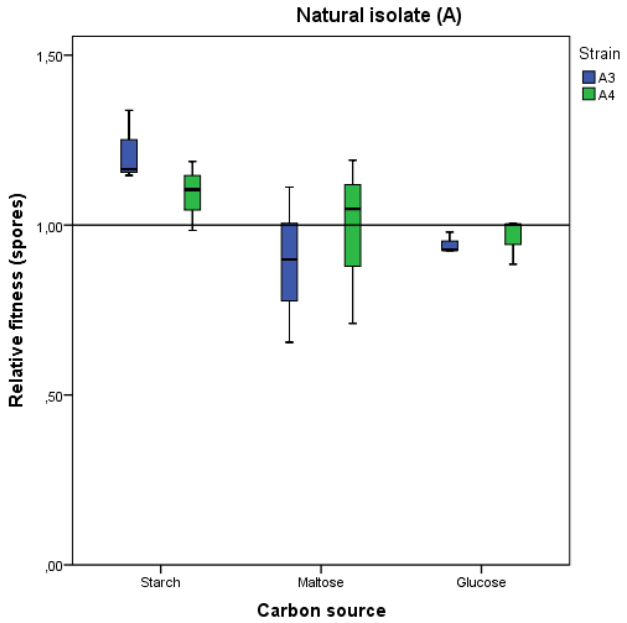

Figure 6a: relative fitness in BM of natural isolate A. A3 performs better on the medium on which it was allowed to evolve, compared maltose and glucose media.

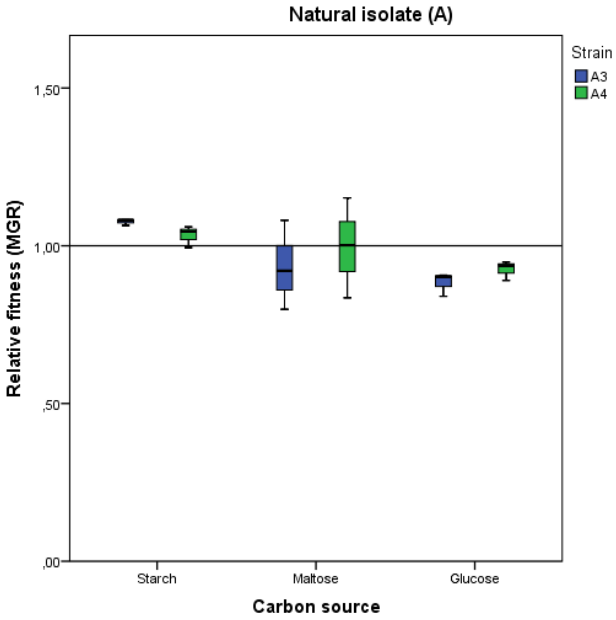

Figure 6b: relative fitness in MGR of natural isolate A. The response in MGR shows a clear correlation to the response in BM.

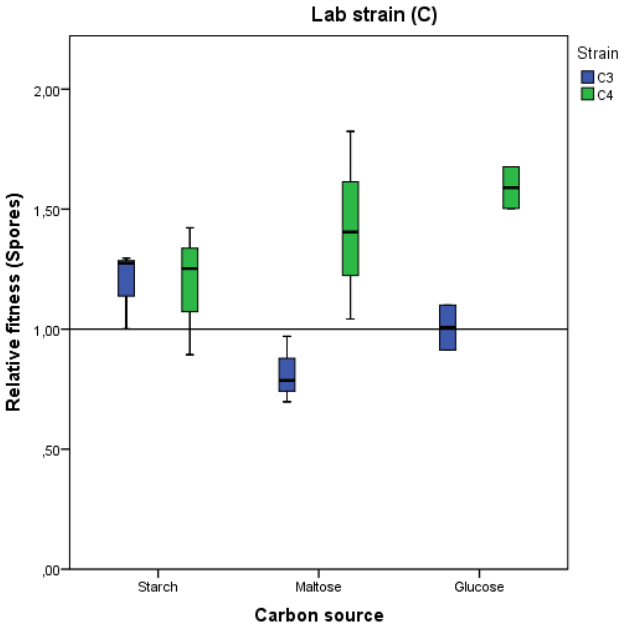

Figure 7a: relative fitness in BM of lab strain C. C3 shows an increase in relative fitness only when growing on starch. C4 performs better over all. This can indicate different methods of adaptation

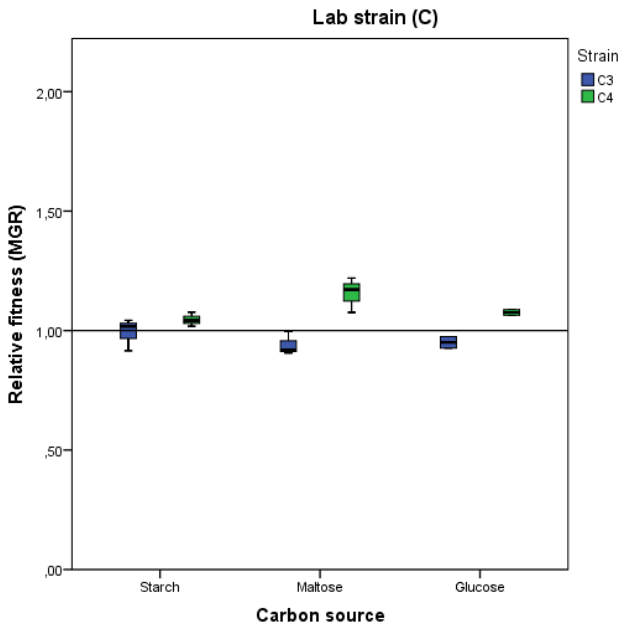

Figure 7b: relative fitness in MGR of lab strain C. As in the natural isolates the fitness proxy's of BM and MGR show a clear correlation.

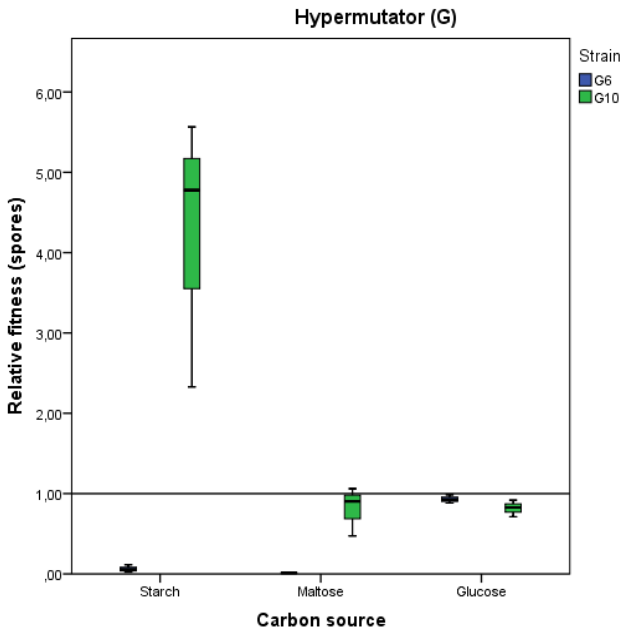

Figure 8a: relative fitness in Bm of Hypermutator strain G. G10 shows the highest specific adaptation to starch while G6 decreased in both BM and MGR.

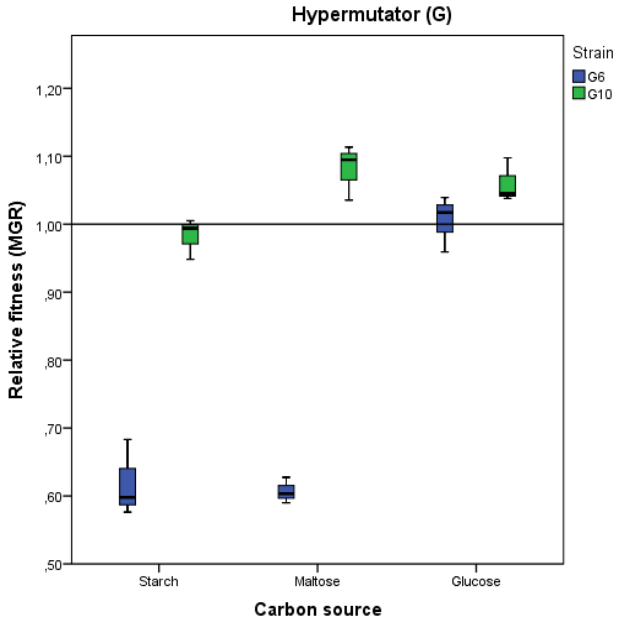

Figure 8b: relative fitness of Hypermutator (G) (MGR)

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Supplementary data

ImageJ

Images were cropped in order to exclude edges from analysis and color thresholded to include only pixels from spores in the analysis using the same macro for all natural isolates. Thresholding values: Hue: 25, 39, stop; Saturation: 2, 255, pass; Brightness: 0, 210 pass.

Example:

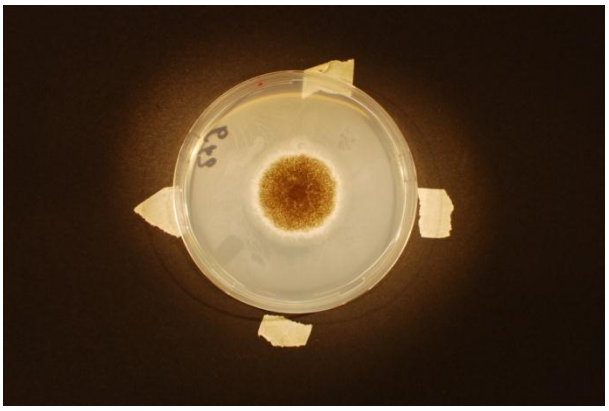


Figure S1: Input image for ImageJ

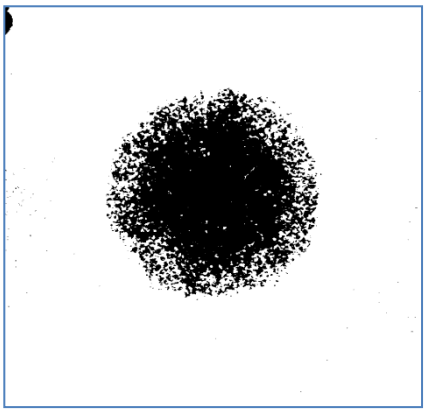
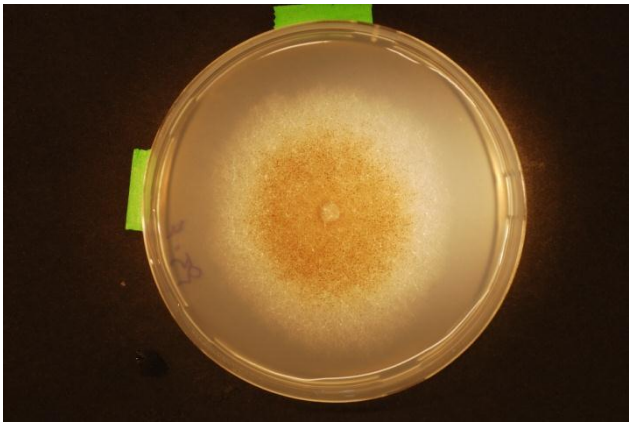


Figure S2: Pixels that were analyzed are highlighted in black. (Measure = IntDen)

As the macro needs to be usable for a substantial series of images, the parameters used must be averaged over a range of values. However, this method remains sensitive to changes in both spore color and number as is shown below:



Figuur S3: Input image for ImageJ

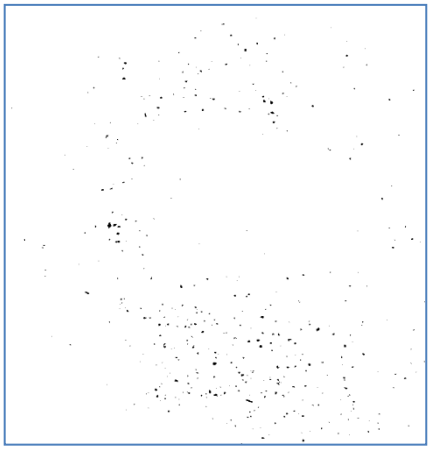


Figure S4 : Pixels that were analyzed are highlighted in black. Note the underestimation of spore number.

Therefore a different macro has been used for each set of images from the selection experiment. Setting the thresholds introduces an unavoidable bias that is particularly troublesome when comparing absolute values between sets of images that were analyzed with a different macro.

The IntDen values obtained by ImageJ were tested for normality (see image S5 and S6). Values were Log(10) transformed to compress the graphs.

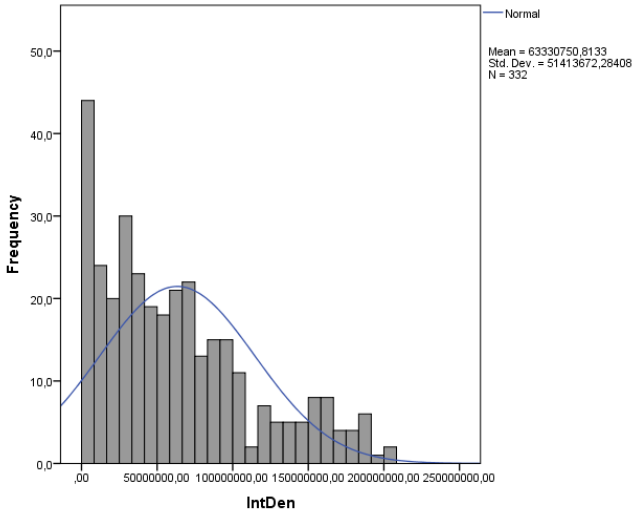


Figure S5: IntDen values from ImageJ

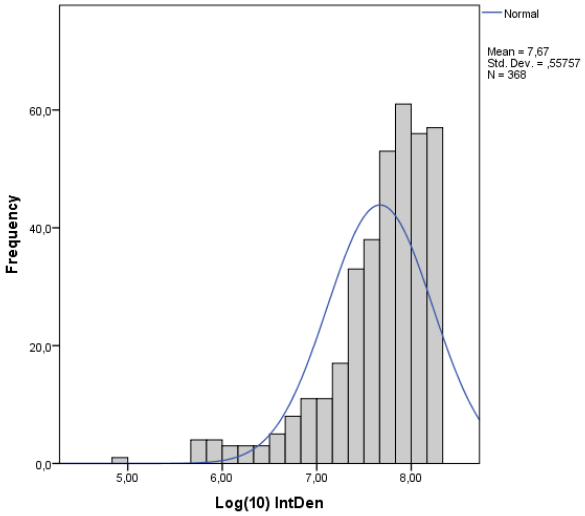


Figure S6: Log(10) transformed IntDen values from ImageJ

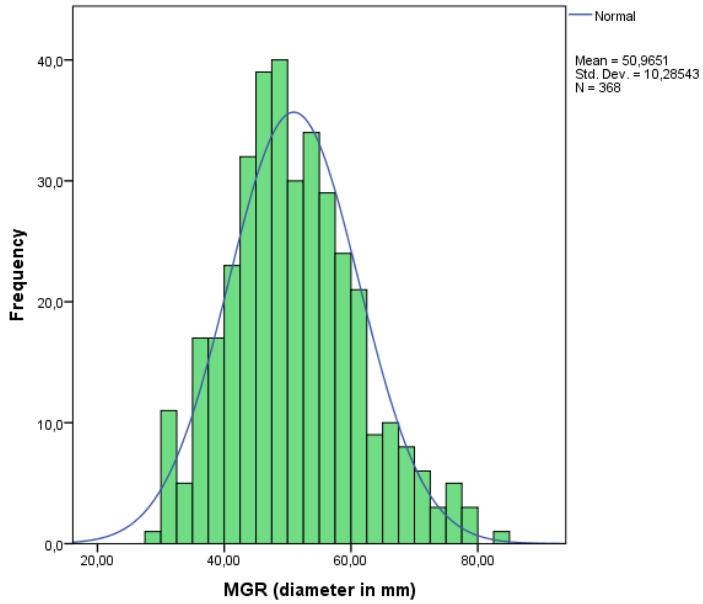


Figure S7: Distribution of MGR data.