Yeast lines selected for non-quiescence show increased protein content

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ABSTRACT

Increasing demand for food production requires improvements as well as openness to new sources of protein. Single Cell Protein derived from microbes has been intensively studied as a supplement for traditional sources of proteins, both for animal feed and direct human consumption. Food grade yeast, including *Saccharomyces cerevisiae*, has already been successfully used in industry. Here, we describe an artificial selection experiment that resulted in isolating *Saccharomyces cerevisiae* lines with a heritable phenotype characterized by significantly higher total protein content. Compared to the ancestral population, the average increase in protein content for all the evolved lines containing multiple-evolved clones was 5.4 g per 100g of dry mass (~15% of the total protein content). However, we also obtained specific clones with a total protein content increase of 9.3 g/100g dry mass (increase by 24.6%). Whole genome sequence analysis of mutations acquired by these clones allowed us to hypothesize about the role of the amino acid signaling pathway (SPS) disorders as a genetic base of the increased amino acid content. The proposed method is based on gradient fractionation of starved haploid yeast cells of different density, which reflects their physiological state regarding quiescence. We found a positive correlation in the fraction of non-quiescent cells in the starved populations and their amino acid content. Our method does not require any genetic modification. We believe that it can be successfully applied to other *Saccharomyces sp*. in order to increase the amino acid content stored in their cells. Beyond its implications for applied science, knowledge of the quiescent state in yeast is of fundamental importance to our understanding of the genetic basis of the G0 state in eukaryotic cells.

keywords: ***Single Cell Protein, SPS pathway, non-quiescent cells, non-GMO yeast***

1. INTRODUCTION

Humans and animals consume protein as a source of nitrogen and amino acids, from which they build new structural and functional proteins (e.g. enzymes and hormones) that enable them to survive. In extreme conditions, proteins may also be used as a source of energy. The nutritional value of a protein is determined by its amino acid composition. Twenty amino acids are commonly found in dietary protein, several of them (i.e. phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, and lysine) are exogenous and cannot be synthesized by humans and other animals. These essential amino acids have to be supplied through the diet (Wu, 2009). Moreover, all the amino acids should be provided in the right ratio and even when only one of the essential amino acids is not provided in adequate amounts in the diet, protein synthesis is limited by the rate at which this limiting amino acid is available (Wolfe, Baum, Starck, & Moughan, 2018).

Increasing demand for food production requires improvements as well as openness to new sources of protein, both for animal feed and direct human consumption (Boland et al., 2013). The main sources of protein are cereals, meat, eggs, and dairy products. The protein content in cereals is usually in the range from 8% to 11% but it can sometimes vary from 6% to 20%, depending on the variety and growing conditions (Koehler & Wieser, 2013). Raw meat (beef, pork, lamb, and poultry) contains approximately 20% of protein and so does fish meat (Bohrer, 2017). The average protein content in hen egg is about 12% of fresh mass, while water content is about 75% (Abeyrathne, Lee, & Ahn, 2013). Although bovine milk contains only about 3% of protein, milk and dairy products are excellent sources of valuable protein, compared to plant-based milk alternatives, which have lower protein content and are a worse source of essential amino acids (Chalupa-Krebzdak, Long, & Bohrer, 2018).

Protein can also be provided through the consumption of various microbes and algae, preferably those which contain ~30% protein in their biomass and which can provide a healthy balance of essential amino acids. Microbial protein is generally referred to as *Single Cell Protein* (SCP). In addition to their direct use as SCP, microbes are used to upgrade the protein content or quality of fermented foods (Bourdichon et al., 2012). Although microbial protein constitutes a relatively small proportion of current human nutrition, the growing global demand for protein is likely to make SCP increasingly important (Suman, Nupur, Anuradha, & Pradeep, 2015). However, SCP is currently produced from a limited number of microbial species, particularly when considering human consumption. *Saccharomyces cerevisiae*, the brewer's yeast, is one of the most important microorganisms used for this purpose (Ritala, Häkkinen, Toivari, & Wiebe, 2017). In addition, *Saccharomyces cerevisiae* is accepted for human and livestock consumption and used a probiotic (Additives & Feed, 2014) which serves as a nutritional supplement rich in B-vitamins (Bekatorou, Psarianos, & Koutinas, 2006; Ritala et al., 2017). For more than a century, spent brewer's yeast has been consumed by humans as *yeast extracts* such as Marmite® (Unilever and Sanitarium Health Food), Vegemite® (Bega Cheese Ltd.), Cenovis® (Gustav Gerig AG), and Vitam-R® (VITAM Hefe-Produkt GmbH). Yeast extracts used in the form of pastes are valuable components of both vegetarian

and conventional diets, as they contain all the essential amino acids, especially large amounts of lysine, valine and isoleucine along with Group B vitamins (Ritala et al., 2017).

Yeast was domesticated over seven thousand years ago and since then has been used for fermentation processes during production of alcoholic beverages and bread leavening (Libkind et al., 2011; Mortimer, 2000). *Saccharomyces sp.* is used worldwide in breweries, wineries, and distilleries for the production of beer, wine, distillates, and ethanol (for starter cultures); for the production of bread and baking products; and during production of specific types of fermented foods like cheese, sourdoughs, fermented meat and vegetable products, vinegar, etc. Discovering the essential role of *Saccharomyces cerevisiae* in alcoholic fermentation (Pasteur, 1858) started a profound interest in broadly understood yeast biology, lasting until today. However, it was not so long ago that it was discovered that the stationary phase population of the haploid *S. cerevisiae* consists of at least two types of cells, which differ in many features (Allen et al., 2006). Quiescent cells (Q) are denser, unbudded daughter cells formed after glucose exhaustion. They synchronously reenter the mitotic cell cycle, suggesting that they are in a G0 state similar to the G0 cells of higher eukaryotes such as stem cells, neurons and eggs. Non-quiescent (NQ) cells are less dense, heterogeneous, and replicative older. Asynchronous NQ cells rapidly lose their ability to reproduce while starved. During fractionation of the starved population in density gradient, Q cells form the lower band, while NQ cells can be found in the upper band. The fraction of Q and NQ cells can be easily isolated based on the differences in cell density (Aragon et al., 2008).

Today, unicellular *Saccharomyces cerevisiae* is not only one of the best known model eukaryotic organism, widely used in basic and applied science research (Duina, Miller, & Keeney, 2014; D. M. Wloch-Salamon, 2014), but also the most common food grade yeast (Ritala et al., 2017). The short growth times of yeast, leading to rapid biomass production together with inexpensive and easy cultivation and harvest, mark its advantages as Single Cell Protein compared with conventional sources of protein (Ravindra, 2000; Suman et al., 2015). Food grade *Saccharomyces cerevisiae* as an alternative source of protein has already been successfully used in industry and might play a substantial role in covering the increasing nutrition demands of the world, especially in areas with low agricultural production and rapidly increasing population (Ritala et al., 2017).

In order to obtain superior industrial yeast strains, several strategies can be applied (for a review of methods see (David & Siewers, 2015; Steensels et al., 2014). Firstly, the existing natural diversity can be explored by genotyping and phenotyping isolated feral strains or strains from yeast collections, to select the most interesting variants (Francisco A Cubillos, 2016; Natesuntorn, Phaengthai, Sompugdee, Sakulsombat, & Sriroth, 2019). Secondly, yeast diversity can also be generated artificially. For this, methods based on genetic engineering, where a recombinant piece of DNA is transformed in a target strain to confer a specific, industrially relevant phenotype to this strain, can be very efficient (Steensels et al., 2014; D. Wloch-Salamon, Majewska, Pajak, Pawlik, & Plech, 2013). However, these techniques genetically modify yeasts, currently limiting their use in food or beverage fermentations. At present, legislation restricts the use of GMOs and GMMs in foods to a much larger extent in the European Union than in the United States and other countries. The majority

of microbial strains used in the EU food industry market are generally obtained by uncontrolled genetic changes (i.e. random mutagenesis, dominant selection, adaptive evolution, natural conjugation, and transformation) and are therefore considered *non-genetically modified*, while microbial strains obtained by controlled genetic techniques (i.e. recombinant DNA) are generally not accepted. Of the huge amount of studies conducted to improve *S. cerevisiae* strains for food production, only a few have been approved for industrial use (Ritala et al., 2017). The public perception of GMOs also varies considerably around the world. In Europe, they are mostly considered to be unsafe for human consumption, while in other countries their consumption is partially accepted. To be used in foods, GMOs and GMMs must pass several extensive tests and meet legal regulations (Ritala et al., 2017). Finally, there are also multiple strategies considered to not involve genetic modification, which induce genetic diversity in a single strain or shuffle the genomes of multiple strains. Strains resulting from these strategies can be freely used in industrial fermentations. For yeasts, most techniques are based on: (1) sexual or (2) asexual hybridization; (3) global transcription machinery engineering, based on obtaining a library of random mutated versions of a global transcription factor by simultaneous multiple gene modification (Alper & Stephanopoulos, 2007); (4) mutagenesis, either chemical (EMS) or physical (UV light); and (5) adaptive (directed or artificial) evolution in response to certain cultivation or growth conditions.

In the techniques based on *artificial evolution*, a population of cells is grown under continuous selection for the phenotype of interest for many generations (counted as cell divisions). This method , also called *directed evolution* can be also combined with the use of mutagens and/or sexual hybridization within the evolving population(s), in order to increase the genetic and phenotypic variation that selection can act on. Over time, random mutants will arise in the population and if a specific mutation (or mutations) endows a cell with a fitness advantage, this variant will be selected and enriched for in the population. Because of the short generation time (~90 min.) and easy manipulation and cultivation of microorganisms in the laboratory, evolutionary engineering is a feasible route to generate yeast strains with improved phenotypes in a relatively fast fashion (Elena & Lenski, 2003; Buckling et al., 2009). Directed evolution has proven to be a valuable tool to create yeast strains with specific, improved characteristics (Sauer, 2001; Tilloy, Cadiere, Ehsani, & Dequin, 2015).

Here, we describe a new method to obtain yeast strains with increased protein content, based on NQ cell fraction enrichment during an artificial selection experiment. The evolved *Saccharomyces cerevisiae* lines contained ~4 times more NQ cells and ~15% more total protein in comparison to the ancestral strain. During the experiment, yeast lines acquired several spontaneous mutations. Analysis of the mutation spectrum in clones derived from the final lines revealed multiple mutations in genes constituting the amino acid signaling (SPS) pathway. We found a positive correlation between the quantity of the NQ cell fraction in glucose-starved populations and amino acid content for all the evolved lines and isolated clones. These results allow us to hypothesize about a mechanism responsible for coupling an easy to detect phenotype, i.e. the NQ cell fraction, with amino acid content increase. As far as we know, a method based on population density gradient fractionation for obtaining *S. cerevisiae* strains with heritable, significantly increased protein content has not been proposed so far.

2. MATERIALS AND METHODS

2.1. Serial enrichment experiment

The experimental selection that we performed was described previously (D. M. Wloch-Salamon, Tomala, Aggeli, & Dunn, 2017). In brief, we used a derivative of the laboratory haploid strain s288C (*Mat α, ura3::KanMX4*) (F. A. Cubillos, Louis, & Liti, 2009). The ancestral clone (t0) was grown on YPD medium to the stationary phase population and then diluted and spread on three plates (with initial density ~$2 \cdot 10^5$ cells/ml). The plates were then incubated in 28^{o}C for 3.5 days and subsequently all the cells were harvested. Each population grown on a separate plate was then separately fractionated in density gradient to obtain two cell fractions: upper (NQ) and lower (Q), which differ in density (Allen et al., 2006). From each of the three plates, we obtained distinct fractions of NQ cells that were founders of the 3 initial (t1) experimental lines (I, II, III). We progressed the experiment by using only NQ cells from the fractionated cultures, diluted them, and plated on YPD medium at an initial density of ~$2 \cdot 10^5$/ml. The plates were incubated for 3.5 days, during which the population re-grew to the stationary phase density and then starved. Next, the grown populations were harvested and fractionated in density gradient. We carried out 30 cycles of growth, starvation and fractionation, which as we determined was equivalent to ~300 generations (cell doublings). From the final 30th cycle, we obtained 3 final (t30) lines (I, II, III). From each final population, we also isolated 15-16 *single clones* for further analysis. Lines and clones from different time points of the experiment were frozen in -70^{o}C for further investigation.

2.2. Fractionation, isolation and quantification of less dense (non-quiescent) cells

To quantify NQ cells, we replicated the main experimental conditions as follows. First, the stored populations and clones from time points t0, t1, t30 were thawed. Then, each sample was transferred to liquid YPD medium and incubated overnight to reach the stationary phase. On the next day, each sample was plated on YPD medium in the initial density ~$2 \cdot 10^5$ cells/ml and incubated in 30^{o}C for 3.5 days. All yeast culture was then scraped from the plate and diluted in 10 ml of 50 mM Tris, pH 7.5. Next, a slightly modified standard method (Allen et al., 2006) was applied to obtain fractions of population cells that differed in density. In brief, we mixed Percoll and 1.5 M NaCl, 9:1 v/v, and 4 ml of this solution was centrifuged in an angular rotor (MPW Med. Instruments model MPW-352R) at 10078 g for 20 mins. Then, 2 ml of the yeast culture washed from the plates were spun down, placed on top of the gradient and centrifuged in a swinging-bucket rotor for 60 mins at 417 g. We carefully removed the upper (NQ) and lower (Q) cell fractions into separate tubes using pipettes. The quantity of each of the NQ fractions for each population was assessed based on the spectrophotometric OD measurements and calibration curves equations (for NQ cells: y = OD value x $1x10^8$; for Q cells: y = OD value x $9x10^7$). The fractionation procedure was repeated at least two to four times for each analyzed sample (both lines and selected clones). We measured the proportion of NQ cells in: (i) the populations derived from the 3 experimental lines (t30: I; II, III) and in the same lines after the first fractionation (t1: I, II, III); and (ii) in the selected 6 evolved clones (t30) and the ancestral clone (t0) (Table 1). The NQ cell fraction was divided by the NQ fraction in the populations derived from the

ancestral clone (t0), which was included as a reference in every fractionation round. The one-way ANOVA, the post-hoc Tukey test, and the t-test were used for statistical analysis of the difference in NQ cells fractions between samples.

2.3. Whole genome sequencing

We used the available whole genome sequencing data, described in detail in our previous paper (D. M. Wloch-Salamon et al., 2017). For the purpose of this project, we used sequences of seven clones: 2 clones isolated from each of the experimental t30 lines (1_SSY1c; 2_SSY1c; 3_SSY1c; 4_PTR3c; 5_SSY5c; 6_NO SPSc) and the ancestral clone (7_ancestral). We chose 5 clones with a mutation in one of the genes from the SPS pathway, one clone without any such mutations and an ancestral clone as reference (Table 1). Presence of mutations in the SPS pathway (*SSY1 (Gly492Arg); SSY1 (Glu526fs); SSY1 (Tyr747*); PTR3 (Asp263fs); SSY5 (Glu155fs*)) was confirmed by Sanger sequencing. Details of this procedure and results are in supplementary files (Sup.1).

2.4. Analysis of the amino acid content

Strains were transferred from the -70°C deep freeze to a YPD agar plate and grown at 30ºC for 2 days. After that, yeast strains were cultured in standard minimal media supplemented with uracil in 30ºC, in 100 ml tubes, for 3 days, to reach the stationary phase. Then, the culture was washed out 4 times using deionized water, deep-frozen in -70°C and lyophilized. Amino acid analysis was performed according to the method of Moore and Stein (Davidson, 2003; Smith, 2003). Lyophilized samples were hydrolyzed in liquid 6M HCl containing 0.5% phenol at 110ºC for 24 hours under an argon atmosphere. The hydrolysates were lyophilized, dissolved in appropriate volume of dilution buffer (sodium citrate buffer, pH 2.2) and filtered through a 0.45 µm syringe filter before inserting to the amino acid analyzer. Amino acids were determined by ion-exchange chromatography with a strong cation ion-exchanger and sodium-citrate elution buffers system, followed by post-column derivatization with ninhydrin and spectrophotometric detection at 570 and 440 nm, according to the standard protocol of the manufacturer (acid analyser AAA400). Sulphur-containing amino acids were analyzed as oxidation products obtained by performic acid oxidation, followed by a standard hydrolysis procedure with HCl. The amino acid standard solution (Sigma, USA) was used for calibration of the amino acid analyzer. Each powdered sample was hydrolyzed in duplicate and each hydrolysate was analyzed chromatographically, also in duplicate. Evaluation of the acquired data was performed using the software of the chromatographic device (CHROMuLAN, Pikron, Czech Republic). Tryptophan was not determined, as it is destroyed during acid hydrolysis; asparagine and glutamine turn to aspartic acid and glutamic acid, respectively, and are determined in these forms. Planned contrasts tests were used for statistical analysis of the difference in amino acid content between the evolved and the ancestral populations. We assessed amino acid content in: the t30 lines (I, II, III); in t0 clone; and in clones isolated from t30 lines (Table 1). For each sample, there were 4 measurements of 19 amino acids (including methionine and cysteine). These results fed the quantification of the average total protein content. We conducted multiple measurements (up to 8) of the 17 (non-sulphur containing)

amino acids (without methionine and cysteine). These results (shown in Tables 2 and 3) were used to quantify each amino acid.

Table 1. List of the clones analyzed for amino acid content and NQ/Q cell balance. NQ cell proportions show the average of 2 measurements.

CLONE NAME	Derived from the line	AQUIRED MUTATIONS (SNV) in SPS pathway	AQUIRED MUTATIONS (SNV) other mutations	NQ cells increase in starved population (relative to ancestral clone set to 1)
1_SSY1c	III	***SSY1 (Gly492Arg)***	*ECM21(Agr106*)* ***SIR2(Cys469*)*** *IRA2(Gln1365*)* *MSN1(Lys86Thr)*	3.66
2_SSY1c	I	***SSY1 (Glu526fs)***	*ECM21(Agr106*)* ***SIR4(Ser154*)*** *IRA2(Gln1365*)* *MSN1(Lys86Glu)*	4.83
3_SSY1c	II	***SSY1 (Tyr747*)***	*ECM21(Agr106*)* ***SIR4(Ser57*)*** *RA2(Gln1365*)* *MSN1(Lys86Glu)* *MTC5(Gly624Val)*	3.94
4_PTR3c	III	*PTR3 (Asp263fs)*	*ECM21(Agr106*)* *IRA2(Gln1365*)* *MSN1(Lys86Glu)* *DEP1(Gln17Lys)* *PHO89(Ile74Phe)* *SEH1(Glu189Lys)* *URA2(Gly447Asp)* *YLR108C(func YLR108C:p.Met1?)*	3.17
5_SSY5c	I	***SSY5 (Glu155fs)***	*ECM21(Agr106*)* ***SIR4(Gln595fs)*** *IRA2(Gln1365*)* *MSN1(Lys86Glu)* *SGV1(Asn587Asp)*	3.35
6_NO SPSc	II	-	*ECM21(Agr106*)* ***SIR4(Ser57*)*** *IRA2(Gln1365*)* *MSN1(Lys86Glu)*	4.29
7_ancestral		-	-	1

3. RESULTS:

3.1 Significant increase of the NQ cell fraction in experimental lines and clones

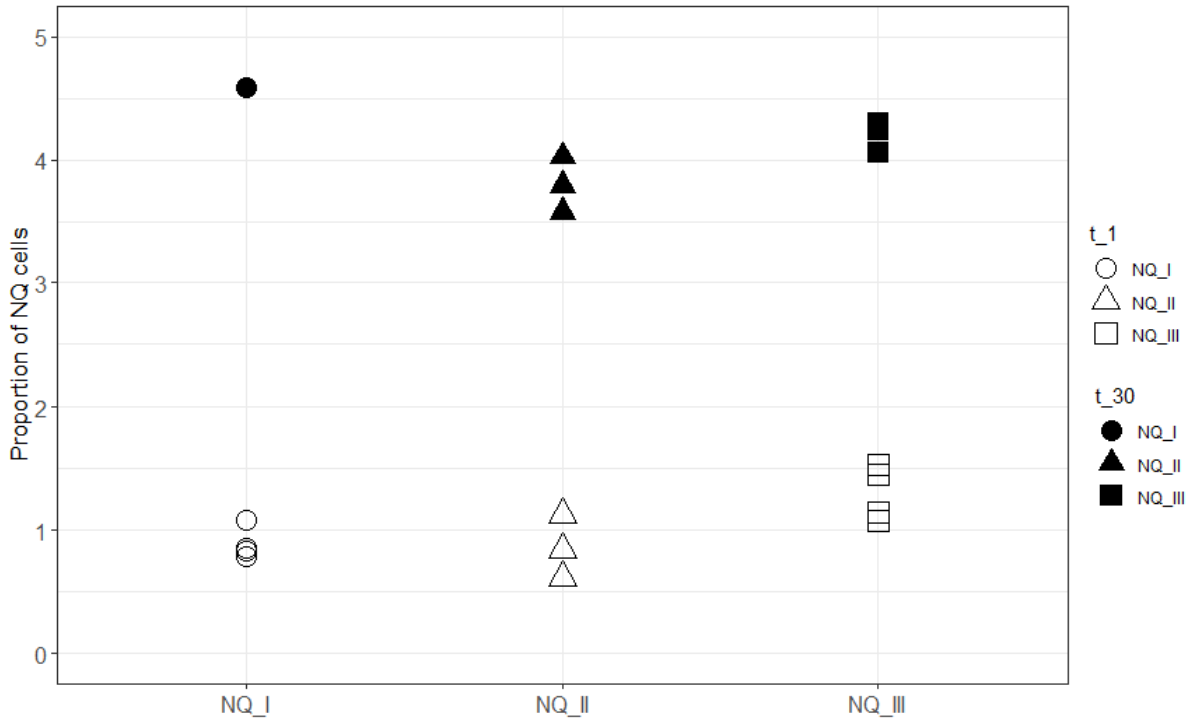
There was a significant increase of the NQ cell proportions in the starved populations, both derived from the experimental lines (t30:I, II, III) (Fig.1) and the populations derived from the selected evolved clones (Table 1).

Lines: The average proportions (±SD) of NQ cells (relative to the populations derived from the ancestral clone t0, used as a reference) at the first fractionation (t1) and at the final fractionation (t30)

were: I = 0.89 ± 0.12 and 4.58; II = 0.86 ± 0.21 and 3.80 ± 0.18; III = 1.30 ± 0.19 and 4.19 ± 0.10 for the respective populations (Fig.1). A one-way ANOVA showed a significant increase in NQ cell proportions between t1 and t30 ($F(1, 16) = 437.93$, $df = 12$, $P < 0.0000001$). A post-hoc Tukey HSD test showed a significant difference between each of the experimental lines isolated from t30 and t1. Hence, NQ cells constituted on average 18.46% ± 1.73 and 70.21% ± 4.501 of the population at the beginning and at the end of the experiment, respectively.

Clones: The average proportion of NQ cells in the populations derived from the selected clones was 3.87 ±0.67 times higher than in the population derived from the t0 ancestral clone (1.0 ± 0.23) (Table 1.). A t-test showed a significant difference between the ancestral clone and the average for the selected 6 clones ($t = -9.273$, $df = 15$, $p < 0.0000001$). NQ cells composed on average 56.16% ± 9.24 of all the cells in the starved populations derived from the evolved clones.

Fig 1. Proportion of NQ cells (relative to the t0 ancestral clone) in the starved population before (t1) and after (t30) experimental evolution.



3.2 Significant increase of amino acid content

Lines: The average increase of total protein content in the cells for each line (t30: I, II, III), relative to that of ancestral population (t0) was 6.46 g ±1.9 g, 6.0 g ±0.56 g, and 3.82 g ±1.19 g for 100 g of dry mass, respectively, which on average constituted ~15% of the total protein content. A planned contrasts analysis demonstrated that the difference between the ancestral and all the three evolved lines was significant ($F(3,12) = 14.344$; $p = 0.00028$). A planned contrasts analysis of each of the 19 selected amino acids (aspartic acid and asparagine, and glutamic acid and glutamine measured together) from the evolved lines populations showed a significant increase in all the cases compared

to the ancestral population, except for cysteine and methionine, the content of which significantly decreased in the evolved lines (Table 2). The average increase of the content of the seven exogenous amino acids (compared to the ancestral population content t0 = 15.27 ± 0.67) was 2.96 g ±0.81 g, 2.91 g ±0.21 g, and 1.9 ±0.41 g per 100 g of dry mass, respectively. Detailed results are shown in Table 2.

Clones: Planned contrasts demonstrated that the population derived from 4 out of the 6 evolved clones differed significantly in total protein content from the ancestral population ($F(6, 21) = 8.512$; $p = 0.00009$) (detailed results are shown in Table 3). Clones with no mutation in the SPS pathway and the clone with the mutation 3_SSY1c gene did not differ in total protein content compared to the ancestral population. The average total protein content increase for all the clones was 9.3 g/100g dry mass, which was greater by 24.6 % compared to the ancestral population. There was a significant difference in the content of the 19 selected amino acids (aspartic acid and asparagine, and glutamic acid and glutamine measured together) from the evolved clones compared to the ancestral population ($F(6, 546) = 2.9326$, $p = 0.00798$). Detailed results of the planned contrasts analysis are shown in Table 3.

Table 2. Amino acid content of the ancestral (t0) and *evolved lines (t30 I, II, III)* (g/100g lyophilized cells). Means ± SD are shown. The up and down arrows indicate a statistically significant increase or decrease of a given amino acid content in the selected vs. ancestral population. *# sign denotes exogenous amino acids.*

Total amino acid content (total protein)				
	Ancestral Line	Line I	Line II	Line III
Total amino acid content	37.84 ± 1.73	44.30 ± 2.20 ↑	43.84 ± 0.65 ↑	41.66 ± 1.19 ↑
Alanine	2.39 ± 0.09	2.64 ± 0.11 ↑	2.65 ± 0.05 ↑	2.51 ± 0.08 ↑
Arginine	2.09 ± 0.07	2.72 ± 0.12 ↑	2.63 ± 0.04 ↑	2.66 ± 0.09 ↑
Aspartic acid + Asparagine	4.09 ± 0.24	4.70 ± 0.22 ↑	4.69 ± 0.07 ↑	4.42 ± 0.15 ↑
Cysteine	0.58 ± 0.02	0.48 ± 0.03 ↓	0.40 ± 0.01 ↓	0.47 ± 0.07 ↓
Glutamic acid+ Glutamine	4.92 ± 0.23	6.16 ± 0.22 ↑	6.03 ± 0.13 ↑	5.77 ± 0.23 ↑
Glycine	1.83 ± 0.08	2.16 ± 0.10 ↑	2.15 ± 0.04 ↑	2.00 ± 0.06 ↑
Histidine #	0.98 ± 0.05	1.21 ± 0.06 ↑	1.18 ± 0.02 ↑	1.15 ± 0.03 ↑
Isoleucine #	2.00 ± 0.12	2.42 ± 0.12 ↑	2.40 ± 0.04 ↑	2.23 ± 0.06 ↑
Leucine #	2.96 ± 0.21	3.50 ± 0.16 ↑	3.48 ± 0.06 ↑	3.26 ± 0.12 ↑
Lysine #	3.25 ± 0.14	3.99 ± 0.18 ↑	3.91 ± 0.05 ↑	3.73 ± 0.12 ↑
Methionine #	0.77 ± 0.02	0.63 ± 0.04 ↓	0.55 ± 0.04 ↓	0.61 ± 0.07 ↓
Phenylalanine #	1.81 ± 0.10	2.41 ± 0.10 ↑	2.52 ± 0.05 ↑	2.27 ± 0.09 ↑
Proline	1.45 ± 0.07	1.72 ± 0.07 ↑	1.76 ± 0.02 ↑	1.58 ± 0.01 ↑
Serine	1.96 ± 0.09	2.37 ± 0.09 ↑	2.39 ± 0.05 ↑	2.27 ± 0.08 ↑
Threonine #	2.14 ± 0.13	2.60 ± 0.12 ↑	2.62 ± 0.05 ↑	2.52 ± 0.09 ↑
Tyrosine	2.27 ± 0.12	1.81 ± 0.07 ↑	1.78 ± 0.01 ↑	1.66 ± 0.06 ↑
Valine #	2.34 ± 0.10	2.71 ± 0.13 ↑	2.71 ± 0.04 ↑	2.55 ± 0.07 ↑

Table 3. Amino acid content in the ancestral (t0) and *selected evolved clones (t30,Table 1)* (g/100g lyophilized cells). Means ± SD are shown. The up and down arrows indicate a statistically significant increase or decrease in amino acid content of the selected vs. ancestral clone. *# sign denotes exogenous amino acids.*

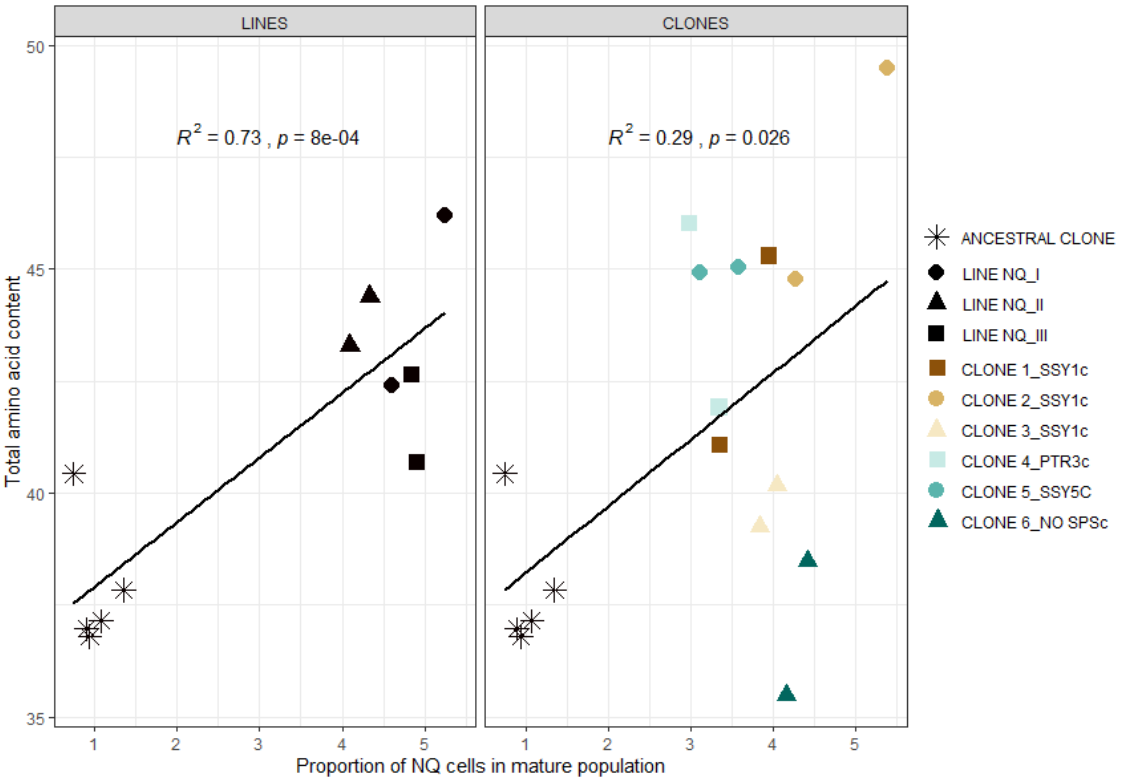
	Total amino acid content (total protein)						
	Ancestral	1_SSY1c	2_SSY1c	3_SSY1c	4_PTR3c	5_SSY5c	6_NO SPSc
Total amino acid content	37.84 ± 1.73	43.18 ± 2.65 ↑	47.13 ± 4.48 ↑	39.70 ± 2.36	43.96 ± 2.49	44.99 ± 1.3 ↑	36.99 ± 2.2
Alanine	2.39 ± 0.09	2.75 ± 0.18 ↑	2.88 ± 0.22 ↑	2.34 ± 0.14	2.76 ± 0.17 ↑	2.64 ± 0.1 ↑	2.24 ± 0.13
Arginine	2.09 ± 0.07	2.73 ± 0.21 ↑	2.83 ± 0.23 ↑	2.57 ± 0.15 ↑	2.60 ± 0.18 ↑	2.77 ± 0.1 ↑	2.18 ± 0.11
Aspartic acid +asparagine	4.09 ± 0.24	4.66 ± 0.27 ↑	5.06 ± 0.38 ↑	4.06 ± 0.28	4.77 ± 0.34 ↑	4.73 ± 0.17 ↑	3.89 ± 0.29
Cysteine	0.58 ± 0.02	1.80 ± 0.01 ↑	0.57 ± 0.03	0.60 ± 0.06	0.48 ± 0.03 ↓	0.48 ± 0.04 ↓	0.64 ± 0.05 ↑
Glutamic acid +glutamine	4.92 ± 0.23	6.46 ± 0.55 ↑	6.91 ± 0.55 ↑	5.94 ± 0.39 ↑	6.28 ± 0.56 ↑	6.87 ± 0.20 ↑	5.49 ± 0.38
Glycine	1.83 ± 0.08	2.20 ± 0.13 ↑	2.40 ± 0.19 ↑	1.98 ± 0.12	2.21 ± 0.13 ↑	2.16 ± 0.07 ↑	1.85 ± 0.11
Histidine	0.98 ± 0.05	1.49 ± 0.07 ↑	1.30 ± 0.10 ↑	1.06 ± 0.06	1.14 ± 0.07 ↑	1.18 ± 0.04 ↑	0.96 ± 0.03
Isoleucine#	2.00 ± 0.12	2.25 ± 0.12 ↑	2.50 ± 0.19 ↑	2.06 ± 0.13	2.35 ± 0.15 ↑	2.43 ± 0.05 ↑	1.98 ± 0.12
Leucine#	2.96 ± 0.21	3.07 ± 0.18	3.40 ± 0.26 ↑	3.04 ± 0.18	3.36 ± 0.31 ↑	3.54 ± 0.15 ↑	2.86 ± 0.13
Lysine#	3.25 ± 0.14	3.60 ± 0.24 ↑	4.13 ± 0.33 ↑	3.42 ± 0.25	3.85 ± 0.25 ↑	3.97 ± 0.11 ↑	3.21 ± 0.14
Methionine#	0.77 ± 0.02	0.76 ± 0.02	0.76 ± 0.05	0.94 ± 0.1 ↑	0.79 ± 0.07	0.71 ± 0.07	0.87 ± 0.06
Phenylalanine#	1.81 ± 0.10	2.03 ± 0.14 ↑	2.21 ± 0.17 ↑	1.84 ± 0.12	2.09 ± 0.16 ↑	2.10 ± 0.08 ↑	1.68 ± 0.12
Proline	1.45 ± 0.07	1.68 ± 0.06	1.76 ± 0.15 ↑	1.49 ± 0.14	1.67 ± 0.10 ↑	1.63 ± 0.10 ↑	1.43 ± 0.13
Serine	1.96 ± 0.09	2.23 ± 0.14 ↑	2.51 ± 0.20 ↑	2.10 ± 0.16	2.27 ± 0.16 ↑	2.36 ± 0.05 ↑	1.93 ± 0.13
Threonine#	2.14 ± 0.13	2.53 ± 0.18 ↑	2.80 ± 0.22 ↑	2.35 ± 0.16	2.57 ± 0.18 ↑	2.63 ± 0.06 ↑	2.12 ± 0.13
Tyrosine	2.27 ± 0.12	1.92 ± 0.16 ↓	1.82 ± 0.17 ↓	1.46 ± 0.14 ↓	2.04 ± 0.4	1.89 ± 0.11 ↓	1.39 ± 0.11
Valine#	2.34 ± 0.10	2.72 ± 0.14 ↑	2.95 ± 0.24 ↑	2.40 ± 0.16	2.78 ± 0.21 ↑	2.75 ± 0.09 ↑	2.29 ± 0.15

3.3. NQ fraction correlates with total protein content in cell

There was a significant positive correlation between the NQ cell fraction (relative to the ancestral clone t0) and the total protein content for all the experimental *lines*; $F(1,9) = 24.465$, $p<0.0008$, $b = 1.44$; $R^2 = 0.73$ (Fig. 2A). There was also a significant positive correlation between the fraction of NQ cells in

the populations derived from the evolved *clones* (Table 1) and the total protein content; F= (1,15) = 6.1268, p<0.0257, b = 1.49; R^2 = 0.29 (Fig. 2B). Correlation between average proportion of NQ cells (relative to ancestral clone t0) and average amino acid content (error bars mark ± SD (standard deviation)) in evolved lines and clones, given separately for each amino acid are shown in Sup.2 file. Correlations only for the clones with mutations in SPS pathway are shown in Sup.3 file

Fig 2. Correlation between the proportion of NQ cells (relative to the ancestral clone t0) and the total amino acid content in the evolved lines (A) and clones (B).



DISCUSSION:

Increased NQ fraction in the population positively correlates with increased amino acid content.

In response to starvation caused by shortage of one or more nutrients, a fraction of cells in a stationary yeast population exit the mitotic cycle and become quiescent (Q). The rest of the cells are called non-quiescent (NQ) (Gray et al., 2004; Valcourt et al., 2012). As a consequence, the stationary phase of haploid yeast culture consists of a characteristic balance of NQ/Q cells. Here, we used a robust but already proven useful method of isolation of Q and NQ cells by fractionation, based on differences in cells density (Allen et al., 2006). During our experimental regime, yeast cells were sequentially subjected to two opposite environmental conditions regarding accessibility of glucose and other nutrients: *feast*, when they multiply; and *famine*, when they starve, when part of the population become quiescent. After successive enrichment only for NQ cells, we obtained evolved cell lines with

a heritable phenotype: increased fraction of NQ cells vs Q cells. This proved that the Q/NQ balance in the population can be modified by selection and we showed that the NQ fraction increased from 18.5% up to 70.2 % of all the cells in the starved population. The increased NQ fraction in the population positively correlated with increased amino acid content (Fig. 2 a, b).

Our method will be useful to obtain yeast with high amino acid content for possible use as dietary supplementation in animal or humans feed. Average protein content of the evolved lines was 43.27 ± 1.15 g/100 g, and was within the range of 30-50 g/100g dry mass typical for fungi used for Since Cell Protein (Ritala et al., 2017; Yamada & Sgarbieri, 2005). Such content of crude protein is comparable with the most valuable plant derived food ingredients: cottonseed meal (40.3% of dry matter), peanut meal (43.9 % dry matter); soybean meal (43.6 % dry matter) (Li, Rezaei, Li, & Wu, 2011). Evolved lines will be especially valuable for producing limiting amino acids for human and animal nutrition including: lysine (8.96 g/100g), threonine (5.96 g/100g), valine (6.14 g/100g) and isoleucine (5.43 g/100g). These values are comparable to the highest protein animal feed or food ingredients: blood meal, casein (Li et al., 2011), and substantially higher than FAO/WHO recommendations for children 2-5 years old (WHO, 1991). Amino acid content was also substantially higher compared to previous results from ethanol distillery yeast strains (Yamada & Sgarbieri, 2005). As is typical for yeast, the ancestral strain's methionine and cysteine contents were low (3.12 g/100g) (Ritala et al., 2017), and content of these amino acids decreased in the evolved lines to 2.42 g/100g. This fulfills the FAO/WHO guidelines for humans older than 10 years (WHO, 1991). Our results also confirm that sulphur amino acids are limiting for the SCP production using yeast.

Spontaneous mutations in SPS pathway may result in increased amino acid uptake

To investigate a genetic mechanism responsible for the observed phenotypic changes (increased NQ fraction as well as increase in protein content), we analyzed the spectrum of mutations acquired during the experiment. The previously sequenced 47 clones from NQ-enriched evolved lines contained multiple (on average 5) mutations (D. M. Wloch-Salamon et al., 2017). There were 63 mutations in 55 genes identified. Four genes: *HSL7, SIR2, SIR4,* and *SSY1* acquired multiple (2 - 5) independent mutations (D. M. Wloch-Salamon et al., 2017), suggesting that they are of potential importance for the obtained pattern of NQ increase. Only 7 (out of 47) clones from the NQ enriched lines did not contain at least one mutation in one of the genes forming the SPS pathway (i.e. *SSY1-PTR3-SSY5*) (D. M. Wloch-Salamon et al., 2017). We found 3 different mutations in the SSY1 gene (one in each experimental line), which suggests that these were independent events. The SPS amino acid sensing pathway consists of three genes, *SSY1, PTR3*, and *SSY5* (Didion, Regenberg, Jorgensen, Kielland-Brandt, & Andersen, 1998; Klasson, Fink, & Ljungdahl, 1999). Yeast cells rely on the SPS-sensing pathway to respond to extracellular amino acids (Didion et al., 1998; Klasson et al., 1999). Sensing of extracellular amino acids through Ssy1p leads to transcriptional induction of genes encoding for amino acid transporters and some other genes, through activation of the transcription factors Stp1p and Stp2p (Boles & André, 2004). There is no evidence so far for a transporter function for this pathway (Boles & André, 2004; Poulsen, Gaber, & Kielland-Brandt, 2008; Poulsen, Wu, Gaber, & Kielland-Brandt, 2005). Instead, this nutrient-induced signal transduction pathway regulates gene expression

by controlling the activity of two redundant transcription factors, Stp1 and Stp2, which activate transcription of amino acid permease genes responsible for transport of amino acids into cells (Andréasson & Ljungdahl, 2002; Boer et al., 2000; Ljungdahl, 2009). Amino acid uptake into null mutants, *Δssy1* and *Δptr3*, is significantly lower than in the wild type strain (Didion et al., 1998; Klasson et al., 1999). Mutations in the *SSY1* gene result in variation in Stp2 transcription level, which positively correlates with increase in amino acid uptake (Poulsen et al., 2008). Constitutive signaling mutants respond to sub-threshold concentrations of inducers (Gaber, Ottow, Andersen, & Kielland-Brandt, 2003; Poulsen et al., 2008). The *SSY1* gene encodes for a non-transporting nutrient sensor and acts as the first step in the SPS pathway (Didion et al. 1998; Klasson et al. 1999), through detection of extracellular amino acids (the next steps involve the PTR3 and SSY5 gene products, see above). All the three of our observed *SSY1* mutations, Tyr747*, Glu526fs, and Gly492Arg, presumably leave the cytoplasmic domain intact (Conrad et al., 2014). The results that we obtained suggest that spontaneous mutations in the SPS pathway may be responsible for the observed increased uptake of the amino acid.

The cell biology of quiescent and non-quiescent yeast cell is important

Beyond its implications for amino acid production, knowledge on the quiescence state is of fundamental importance. A wide range of cells spend most of their life in quiescence, a temporary non-proliferating cellular state. In the wild, unicellular organisms are most frequently quiescent, waiting for signals to re-proliferate, which are as diverse as the presence of specific nutrients, temperature or the level of oxygen (Gray et al., 2004; Lewis & Gattie, 1991). Two decades of research have revealed that quiescent cells are much more complicated that insignificant sleeping G1 cells and are of major importance for many biology processes (Sagot & Laporte, 2019a, 2019b). *Saccharomyces cerevisiae* is proved to be an important model for the eukaryotic quiescent state (Gray et al., 2004; Guidi et al., 2015; Klosinska, Crutchfield, Bradley, Rabinowitz, & Broach, 2011; Rutledge, Russo, Belton, Dekker, & Broach, 2015; Sagot & Laporte, 2019b). Still, the quiescent cell definition and mechanisms of transition to the Q state and its evolutionary consequences are the subject of a constant debate and fascinating discoveries (Krishna & Laxman, 2018; Sagot & Laporte, 2019a, 2019b; Wloch-Salamon, Fisher, & Regenberg, 2017). There have been several scenarios proposed for entering the Q state, however, the nutrient signaling pathway is crucial for the initial stage of eliciting the quiescent state. Appropriate perception of nutrient limitation is critical for a cell to mount an appropriate quiescent program (Broach, 2012). Both the cAMP-dependent protein kinase (PKA) and the target of rapamycin complex 1 (TORC1) pathways play a central role in regulating growth vs. quiescence in response to the quality and quantity of the available carbon source, primarily by stimulating mass accumulation and inhibiting the stress response (Broach, 2012; De Virgilio, 2012). These complex scenarios are described in detail in other excellent reviews and are not the subject of this paper. Here, we only propose possible mechanisms that prevent transition to the Q state based on the constitutive activation of the SPS pathway.

For use in the applied science, we propose a new method of artificial evolution to obtain *S. cerevisiae* strains with an average ~15% increase of total protein content. The proposed method is

based on monitoring an easy phenotypic change (NQ fraction increase) and does not involve any genetic modification nor use of a genetic method. We believe that this method can be successfully applied to all the other *Saccharomyces sp*. and is worthy of further investigation.

Data for this study are available at: *to be completed after manuscript is accepted for publication*

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