GRAS Notice (GRN) No. 604
http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm

ORIGINAL SUBMISSION

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GRAS Notification Program
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October 14, 2015

Re: GRAS Notice for adaptively evolved baker's yeast that prevent acrylamide formation in food and beverages

Dear Dr. Gaynor,

In accordance with provisions Sec. 170.30 (Notice of a claim for exemption based on a Generally Recognized As Safe [GRAS] determination), Renaissance BioScience Corp. hereby submits a notification that adaptively evolved baker's yeast that prevent acrylamide formation in food and beverages are GRAS and therefore are exempt from the premarket approval requirements of the Federal, Food, Drug and Cosmetic Act.

Enclosed are three copies of the GRAS report and one electronic copy.

Sincerely,

Matthew Dahabieh, Ph.D.

Head of Research and VP Business Development
Renaissance BioScience Corp.

GRN 000604

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Matthew Dahabieh, Ph.D.

Head of Research and VP Business Development
Renaissance BioScience Corp.

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FOOD ADDITIVE SAFETY

GRAS Notification

For the use of an Adaptively Evolved Baker's Yeast to Prevent Acrylamide Formation in Food and Beverages

Prepared by

Renaissance BioScience Corporation
Vancouver, British Columbia, Canada

Matthew Dahabieh, Ph.D.
Head of Research and VP Business Development

Dated: October 14, 2015

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GRAS Notice

1. GRAS Exemption Claim

1.1. Name and address of the notifier

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Telephone: (604) 822-6499

Scientific Manager: Dr. Matthew Dahabieh
mdahabieh@renaissancebioscience.com

Regulatory: Dr. Matthew Dahabieh
mdahabieh@renaissancebioscience.com

The research and development of an acrylamide-reducing yeast strain was completed in the research laboratory of Renaissance BioScience Corp., Vancouver, BC, Canada. The technology is based on the adaptive evolution of baker's yeast to constitutively activate its native *ASP3* gene, which encodes a cell wall-associated asparagine-degrading enzyme, Asparaginase II.

1.2. Common name of the substance of the GRAS exemption claim

The subject of this notice is a *Saccharomyces cerevisiae* strain adaptively evolved from a commercial baker's yeast strain. The adaptively evolved strain constitutively expresses its native *ASP3* gene, which encodes a cell wall-associated asparagine-degrading enzyme, Asparaginase II. Recombinant DNA technology was NOT used in the development of this strain. Accordingly, there is no foreign, or non-native *S. cerevisiae* DNA present in the genome of this strain. The novel strain is called **Renaissance BioScience Corp.'s Acrylamide-Reducing Yeast**.

1.3. Applicable conditions of use of the notified substance

1.3.1. Purposes for which the substance is used

Acrylamide (AA) is an industrial chemical used to prepare polyacrylamide polymers used in wastewater treatment, papermaking, ore processing, oil recovery, scientific research, and dye/fabric processing. Despite its widespread use, AA is highly toxic to biological systems, as evidenced by plethora of *in vitro*, *in vivo*, and animal model (rats and mice) studies (1-6). Taken together, these data firmly establish AA—and its active metabolite glycidamide—as toxic chemicals with potent mutagenic, cytotoxic, and neurotoxic potential.

As a result of its demonstrated toxicity in non-human systems, AA was classified by the World Health Organization's International Agency for Research on Cancer (WHO–IARC) in 1994 as a group 2A carcinogen—compounds in this group are listed as '*probably carcinogenic to humans*' based on '*sufficient evidence of carcinogenicity in experimental animals and strong evidence that the carcinogenesis is mediated by a mechanism that also operates in humans'.*

In 2002, AA was shown to occur in a variety of common human foodstuffs as a result of Maillard browning reactions that occur during cooking (7-9). More specifically, AA forms rapidly when the amino acid asparagine reacts with reducing sugars in starchy foods or food products once they are exposed to temperatures equal to or greater than 120 °C (e.g. frying, baking, roasting, etc.). Thus, AA is found in significant quantities in a wide variety of food products including bread (from all grain flours), potatoes, potato products (French fries, potato chips, potato flakes and flour), coffee, cereals, vegetables, etc. (10-19).

As a result of its ubiquity in food products, human dietary exposure to AA is widespread. However, there is currently no scientific consensus as to the direct role for dietary AA in causing human cancers. Although more than 30 epidemiological studies have been completed to date (20), many of these studies reach inconsistent or unclear conclusions, thus only supporting a correlative relationship between high dietary AA exposure and a variety of cancers, especially kidney, endometrial, and ovarian cancers (20).

Despite the lack of scientific consensus on the role of dietary AA in causing cancer, many of the world's governments and regulatory agencies have made the risk assessment and mitigation of dietary AA a significant priority. Indeed, the European Food Safety Authority (EFSA), WHO, U.S. Food and Drug Administration (FDA), California Office of Environmental Health Hazard Assessment (OEHHA) and Health Canada all consider the presence of AA in food to be a major concern. Moreover, both the EFSA[1] and US FDA[2] publish industry focused guidance documents for reducing AA in food, as well as support the adoption of an ‘as low as reasonably achievable’ (ALARA) policy concerning the levels of AA in food.

Currently available methods for AA reduction are based on two basic principles: 1) mitigation of AA formation by food processing practices (limiting cooking time and temperature), and 2) elimination of asparagine as the AA precursor. Importantly, only the second strategy reduces the AA potential of foods, as AA can be formed during end-consumer cooking practices. In both commercially-produced and home-prepared foods alike, AA content increases significantly with cooking time and temperature (21). Thus, asparagine-removal based methods of AA reduction are considered superior as they eliminate the potential for downstream AA formation.

A number of methods are currently available for lowering the AA content of foods. These include commercial preparations of the enzyme Asparaginase isolated from fungi (*Aspergillus sp.*), extensive yeast fermentation (22), applying glycine to dough prior to fermentation (23, 24), dipping potatoes into calcium chloride prior to frying (25), replacing reducing sugars with sucrose (26), general optimization of the processing conditions, such as temperature, pH and water content (25, 27, 28), studies regarding different choices of raw materials (27), and fermentation with lactic acid bacteria (*Lactobacillus sp.*). Furthermore, a genetically modified, low asparagine breed of potato has been recently described (29-31). While all of these listed approaches are effective to some degree, they all suffer from one or more adoption barriers, which them impractical during the manufacture of food products. Such barriers include high cost, limited spectrum of efficacy, effect on organoleptic properties of the food, genetically modified (GMO) stigma, and/or ineffective acrylamide reduction under industrial scale food processing conditions.

Baker’s yeast is an ideal organism to reduce acrylamide in food given that it naturally capable of consuming/degrading the AA precursors, asparagine and reducing sugars. Moreover, yeast is already a natural food ingredient to which humans have a long history of exposure, yeast is already a major ingredient in many of the foods for which AA is a significant problem e.g. bread

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[1] http //ec europa eu/food/food/chemicalsafety/contaminants/toolbox_acrylamide_201401_en pdf

[2] http //www fda gov/downloads/food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/ChemicalContaminantsMetalsNaturalToxinsPesticides/UCM374534 pdf

and leavened snack foods, yeast can be incorporated as a transient treatment to other foods for which AA is an issue e.g. potato products, cereals, snack foods and coffee, yeast is inexpensive to grow and easy to work with, and most commercial food producers have pre-existing experience working with yeast. However, under most conditions common to food processing, yeast's innate cellular machinery needed to degrade asparagine is turned off. Thus, unless used with very specific conditions and times, conventional yeast strains are ineffective at reducing AA.

To circumvent this issue and to effectively reduce acrylamide in foods, Renaissance BioScience Corporation (RBSC) proposes the use of a novel strain of baker's yeast named Renaissance BioScience Corp.'s Acrylamide-Reducing (AR) Yeast**.** This strain was developed by adaptive evolution and classical mutagenesis so as to consistently activate yeast's innate ability to consume the acrylamide precursor asparagine. Indeed, under most conditions common to food processing, the yeast Asparaginase II enzyme needed to degrade asparagine is turned off, thus making conventional yeast strains ineffective at reducing acrylamide. In order to have yeast reduce acrylamide formation by constitutively degrading asparagine, our adaptive evolution and classical mutagenesis strategy was designed to modulate yeast's innate preference to utilize other amino acids before using asparagine. This preference is part of a network of nutrient sensing systems and gene regulatory circuits that permit the differential expression of enzymes required to hierarchically utilize nitrogen. Together, the evolved changes of the acrylamide-reducing yeast enable it to constitutively degrade asparagine when Asparaginase II would otherwise be turned off.

Since RBSC's AR Yeast was developed through adaptive evolution and classical mutagenesis, it does not contain any recombinant, foreign, or non-native *S. cerevisiae* DNA.

1.3.2. Foods in which the substance is to be used

The RBSC AR Yeast is intended for use in food and beverage products. The following food and beverage products are known to contain acrylamide that forms from asparagine reaction during the heating/cooking process. Therefore, they may benefit from the application of the RBSC AR Yeast.

- Grain-Based Food
  - Bread (rye, whole wheat, white, etc.)
  - Toasted breads
  - Cereals
  - Cookies
  - Tortillas
  - Baguettes
  - Doughnuts
  - Bagels
  - Biscuits
  - Pizza crust
  - Crackers
  - Wheat-based snacks
  - Extruded snacks and other snack foods
- Vegetable-based Foods
  - French-fried potatoes
  - Potato chips
  - Formed potato products (e.g. Hashbrowns)
  - Potato-based snack foods
- Beverages
  - Coffee
  - Coffee substitutes

1.3.3. Levels of use of the substance

To prevent acrylamide formation in the variety of food and beverage products that the strain can be applied to, we recommend an approximate range of 0.5 to 5% (on a yeast dry weight basis) as an appropriate level of use for RBSC AR Yeast.

1.4. The basis for the GRAS determination

Through scientific analysis, RBSC has determined that the Acrylamide-Reducing Yeast strain is ‘Generally Recognized as Safe’ (GRAS) for use in food and beverage products. This determination is based, in part, on scientific comparison to conventional baker’s yeast, which is GRAS for use in food manufacturing. This determination is also based on comparison to existing GRAS notifications—to which the FDA has ‘no questions’—for the use of a genetically modified yeast to reduce acrylamide in food (GRN 000422), as well as food uses for other *S. cerevisiae* strains (GRN 000120, GRN 000175, GRN 000350, and GRN 000353). RBSC also asserts that the AR Yeast is functionally equivalent to the parent strain from which it was evolved, except for the constitutive expression of its native ASP3 gene. This gene, which encodes for Asparaginase II, allows the strain to degrade asparagine regardless of growth conditions, thereby reducing acrylamide in food products that incorporate the yeast.

1.5. Statement that the data and information that are the basis for the notifier’s GRAS determination are available for the Food and Drug Administration review and copying

Renaissance BioScience Corp. undertakes that all data and information used in this GRAS determination for use of the AR Yeast strain in food and beverage products will be made available for the FDA to review and copy at the following address:

Renaissance BioScience Corporation
Suite 410 – 2389 Health Sciences Mall
Vancouver, British Columbia, Canada
V6T1Z3

Data will be sent to the FDA upon request.

2. Detailed information about the identity of the notified substance

2.1. Host microorganism

The host yeast strain is an industrial bread yeast strain of the domesticated *S. cerevisiae,* which is widely used in the baking industry.

2.1.1. History of use

The yeast *S. cerevisiae* is found as a commensal organism on our bodies. It is in the air that we breathe and it grows naturally on many foods that we eat regularly. This yeast strain has been used in food production for over five thousand years. Ancient Egyptians, Hebrews and Greeks utilized yeast to produce fermented foods and beverages such as baked goods, wine and beer. Over 2.5 million tons of yeast is commercially produced each year worldwide, the largest volume of any microorganism.

S. cerevisiae serves as a model organism for scientific research for numerous reasons. As a eukaryotic organism, yeast cells are structurally and genetically similar to human cells. They each contain about 6,000 genes, many of which are identical or similar to human genes. Over the last 20 years, researchers have found that yeast genes can be manipulated with relative ease and efficacy. As a result, most of what we know about the cell cycle and human cancer was discovered from research on yeast.

Yeast are very convenient microorganisms to work with in small and large volumes. Yeast needs are very basic, their growth rates are high and predictable, and their proliferation can be controlled and scaled. The primary reason that yeast has become one of the most widely studied and understood organism, is that *S. cerevisiae* is known to be generally non-pathogenic and safe.

2.1.2. General characteristics

Commercial baker's yeast is commonly used in the baking and snack food industry. The production of baker's yeast vegetative cells during manufacturing occurs asexually through budding.

Baker's yeast is used for its ability to ferment dough, thereby leavening and conditioning the dough. In addition, the yeast contributes to the flavor and structure of the final baked product.

Commercial baker's yeast strain is normally grown (manufactured) on cane and/or beet molasses under highly aerobic conditions.

2.1.3. Asparagine catabolism in baker's yeast

Conventional (wild-type) baker's yeast is capable of degrading asparagine so as to utilize it as a nitrogen source for growth **(Figure 1)**. However, this ability is only activated when the yeast are starved for other nitrogen sources such as ammonia, glutamate, and glutamine.

The genes responsible for asparagine degradation are *ASP1* and *ASP3*, which encode an intracellular Asparaginase I and extracellular cell-wall associated Asparaginase II, respectively (32-34). *ASP1* exists as a single copy gene (35), while *ASP3* exists as a quadruple tandem repeat locus (36).

ASP1 has been described to be constitutively expressed, but is predominantly responsible for intracellular utilization of asparagine, rather than extracellular scavenging of asparagine for nitrogen (32, 33). As such, *ASP1* activity alone is not sufficient for yeast to degrade significant amounts of asparagine for the purposes of reducing AA in food. To degrade extracellular asparagine, yeast must express Asparaginase II, however *ASP3* is subject to mechanisms that selectively control nitrogen utilization in yeast, more commonly known as nitrogen catabolite repression (NCR) (37-41).

In general, NCR refers to molecular mechanisms—consisting of sensing systems and transcriptional regulatory circuits—that permit differential gene expression of permeases and catabolic enzymes required to degrade nitrogen sources. More specifically, in cultures grown on multiple different nitrogen source, NCR permits yeast to sequentially select good to poor nitrogen sources (32, 42).

As a result of NCR, extracellular asparagine catabolism by Asparaginase II (ASP3) is usually repressed in wild-type baker's yeast. Indeed, food products affected by acrylamide i.e. wheat, vegetables, and coffee, are rich in a variety of quality nitrogen sources for yeast, including ammonia, glutamate, and glutamine.

2.2. The adaptively evolved baker's yeast

Like other microorganisms, baker's yeast is highly amenable to adaptive evolution. Indeed, the technique has been used widely to modify traits relevant to industrial processes such as winemaking and bioethanol production (Reviewed in 43, 44). Adaptive evolution has also been used to study adaptation response to nitrogen-limiting environments (45, 46) and, more specifically, modify yeast's ability to use nitrogen (deregulation of NCR) for the treatment of wastewater (47).

The RBSC AR Yeast is an industrial baker's yeast strain with the adaptively evolved ability to constitutively degrade asparagine, regardless of growth conditions, thereby reducing acrylamide in a variety of affected food products.

2.2.1. Adaptive evolution and classical mutagenesis

Classical mutagenesis and adaptive evolution refers to the successive or iterative adaptation of an organism to novel growth conditions and/or environments. To accomplish this, random mutations are introduced in an organism, followed by characterization of large pool of variants, and selection of individuals with desirable traits. In this way, artificial selection is used to identify desirable genetic variability accelerated through mutagenesis (48). The technique is commonly used in microbiology to impart or enhance desirable traits in industrially relevant microbes and, more specifically, has a long history of use in the food industry (43, 48-60). Importantly, adaptive evolution does not involve the use of recombinant DNA technology; therefore, organisms created by adaptive evolution are not subject to restrictive GMO food legislation and customer acceptance issues.

2.2.2. Specific adaptive evolution methodology

2.2.2.1. Selection methodology

In order to circumvent the NCR that normally keeps *ASP3* repressed, a classical mutagenesis-adaptive evolution method was used. This method was derived from a study of laboratory yeast that used classical mutagenesis and single-round selection to isolate mutants with derepressed *ASP3* (61). In their study, the authors studied mechanisms of NCR-mediated *ASP3* regulation by selecting for mutant yeast cells able to grow on D-Asparagine as the sole nitrogen source in the presence of a non-metabolizable ammonia analog, methylamine (61).

Like all other known life, *S. cerevisiae* does not utilize D-amino acids. However, the cell wall-associated Asparaginase II (*ASP3*) enzyme is unique in that it can degrade D-asparagine, albeit inefficiently (62). From this process, D-aspartic acid and ammonia are produced, of which only the ammonia can be used for growth—yeast cannot metabolize D-aspartic acid. Importantly, the cytosolic Asparaginase I (*ASP1*) cannot degrade D-asparagine.

Thus, culturing cells in media containing D-asparagine as the sole nitrogen source sets up selective conditions in which the only way the cells can grow is to express *ASP3*, degrade external D-asparagine, and then import the released ammonia. While growth on D-asparagine indicates that *ASP3* is being produced, it must also be expressed in strong NCR conditions in order to be fully derepressed.

To select for cells producing *ASP3* despite the presence of strong NCR signals, it is possible to incorporate methylamine in the media. Methylamine is an ammonia analog that is incorporated into the cell by the same transporters as ammonia but once inside, it cannot be metabolized for growth. Critically however, methylamine does result in strong NCR, equivalent to growth on yeast’s preferred nitrogen source, ammonia (63, 64).

Taken together, growth on D-asparagine as the sole nitrogen source and methylamine sets up conditions in which cells must express *ASP3* to degrade D-asparagine in order to liberate ammonia. However, the ammonia must then compete with methylamine in order to be used for growth by the cell. As such, cells that best de-repress *ASP3*—in spite of the strong NCR signals provided by methylamine—produce the most ammonia, thereby gaining a selective advantage for growth.

2.2.2.2. Adaptive evolution methodology

Using the methodology outlined in Section 2.2.2.1 as a framework, we developed an iterative adaptive evolution strategy to develop the AR Yeast **(Figure 2)**. The goal of this strategy was to slowly increase selective pressure over time so as to build up genetic diversity allowing for full *ASP3* expression—and therefore asparagine breakdown—in otherwise repressive conditions. Importantly, the selective conditions necessary to ensure full de-repression of *ASP3* had to be increased slowly, as the final concentrations of D-asparagine and methylamine used for selection completely prevent the growth of non-adapted yeast. Thus, only the specific combination of classical mutagenesis and adaptive evolution employed was capable of yielding the AR phenotype.

Accordingly, the adaptive evolution procedure we used is as follows:

1. Continuously subculture the commercial parent baker's yeast strain in the presence of media containing D-asparagine as the sole nitrogen source (selective media)
2. Subject cells to UV mutagenesis weekly for a period of time until the growth rate of the cells reaches that of cells grown on non-selective/rich media
3. Continuously subculture the interim evolved cells in selective media now containing methylamine
4. Subject cells to UV mutagenesis weekly for a period of time until the growth rate of the cells in the presence of methylamine reaches that the same cells in the absence of methylamine
5. Isolate individual colonies by plating on selective media containing methylamine
6. Assay selected colonies for the ability to degrade L-asparagine under non-inducing conditions

2.2.3. Asparagine degradation of the Acrylamide-Reducing Yeast

The expression of *ASP3*, and thus the ability of baker's yeast to degrade asparagine, is repressed under most growth conditions. In order to maximize the functionality of the AR Yeast, it was crucial to disrupt the normal NCR mechanisms controlling *ASP3* expression. As such, AR Yeast should degrade asparagine even when grown in nutrient rich media. To test the functionality of the adapted yeast, their ability to degrade asparagine relative to the non-adapted wild-type parent strain was compared.

From the adaptive evolution protocol, an adaptively evolved strain with the desired phenotype (growth on D-asparagine in the presence of methylamine) was identified—RBSC AR Yeast. When this strain was tested for asparagine degradation activity, it exhibited high levels of asparagine breakdown even when grown under nutrient rich (repressive) conditions **(Figure 3)**. Compared to the parent strain—which showed no appreciable activity up to three hours, and only minimal activity afterwards (greater than 60% residual L-asparagine)—a high level of L-asparagine degradation activity was observed in the RBSC AR Yeast strain, even with as little as one hour of assay time (54% L-asparagine remaining at 1 hour). Taken together, **Figures 2 and 3** indicates that the adaptive evolution protocol used successfully altered normal NCR conditions such that the RBSC AR Yeast degrade asparagine even when grown in rich media.

2.2.4. Method of manufacture of the Acrylamide-Reducing Yeast

As it is functionally equivalent to conventional baker's yeast, the RBSC AR Yeast can be manufactured in the exact manner as all yeast used in baking, brewing, or winemaking. Importantly, the RBSC AR Yeast may be manufactured in either a fresh or active dry yeast, as is common to the aforementioned industries (65). A brief description of the manufacturing of RBSC AR Yeast is given in **Figure 4**.

2.2.5. Similarities and differences of the Acrylamide-Reducing Yeast to existing GRAS notifications

The RBSC AR Yeast shares some similarities to an existing GRAS notification for 'Functionally-Enhanced Yeast to Prevent Acrylamide in Heated Foods' (GRN 000422). This notification was filed by Functional Technologies Corporation on January 31, 2012 and closed by the FDA with ‘no questions’ on August 31, 2012.

2.2.5.1. Intended Use

Both the RBSC AR Yeast and the yeast described in GRN 000422 are industrial baker’s yeast strains (*S. cerevisiae*) with an ability to constitutively degrade asparagine, thereby preventing the formation of acrylamide in food products. As such, both yeasts share the same purpose of use (Section 1.3.1), foods in which they are used (Section 1.3.2), and levels of use in foods (Section 1.3.3). Moreover, given that the two yeasts are both industrial baker’s strains, the method of manufacturing is also the same (Section 2.2.4).

2.2.5.2. Strain Development

Despite the fact that both the RBSC AR Yeast and the yeast described in GRN 000422 are industrial acrylamide-reducing baker’s yeast strains (*S. cerevisiae*), they differ markedly in their method of development.

As noted in Section 2.2, the RBSC AR Yeast was developed by adaptive evolution and classical mutagenesis. Moreover, as described above, the technique is commonly used in microbiology to impart or enhance desirable traits in industrially relevant microbes and, more specifically, has a long history of use in the food industry (43, 48-60). Importantly, adaptive evolution does not involve the use of recombinant DNA technology; therefore, organisms created by adaptive evolution do not contain foreign or recombinant DNA and, thus, are not considered to be GMO according to European Union definition followed by many jurisdictions worldwide.

In contrast, the yeast described in GRN 000422 was developed using recombinant DNA technology. More specifically, the notifiers and the FDA note that, “the host strain [an industrial baker’s yeast strain (*S. cerevisiae*)] was transformed with three copies of the *S. cerevisiae* ASP3 gene encoding for Asparaginase. The resulting genetically modified baker’s yeast overexpresses Asparaginase [thereby allowing it degrade asparagine and prevent acrylamide formation in foods]”. It was also noted that, “sequencing analyses confirmed that the inserted DNA sequences in the modified baker’s yeast were all obtained from *S. cerevisiae* and that no foreign genetic material was incorporated into the yeast genome”.

3. Determination that the use of the Acrylamide-Reducing Yeast strain in food and beverage products is exempt from the pre-market approval requirements of the act because such use is GRAS

3.1. Safety assessment of the host strain

3.1.1. Safety of baker's yeast

The host yeast strain is an industrial strain of *S. cerevisiae* known as baker's yeast that is commonly used in commercial bread and leavened baked goods production. *S. cerevisiae* is an organism that has an extensive history of safe use. It has been used for millennia in fermentation processes, such as bread leavening and wine or beer production. Additionally, as yeast is found naturally on the skins of grapes, it is responsible for spontaneous fermentation of grape juice (66). The Bureau of Alcohol, Tobacco and Firearms rates yeast or yeast cultures grown in juice of the same kind of fruit (here grapes) as permitted material added in the production of natural wines (27 CFR 24.176).

S. cerevisiae is considered GRAS through its use in the brewing, baking and winemaking industry. Its genome has been sequenced, and it has been determined that the yeast is free of known pathogenicity traits. Moreover, genetically modified yeast strains have previously been granted GRAS status by the FDA for various purposes. These include yeasts for winemaking (ML01–GRN 000120, ECMo01–GRN 000175, and P1Y0–GRN 000350), nutritional benefits (High selenium yeast– GRN 000353), and reducing acrylamide in food products (Acrylamide-Preventing Yeast–GRN 000422).

In the 27th report of The Scientific Committee for Human Food of the European Community the authors stated that *S. cerevisiae* has a safe history of use in food and belongs to a species that is known not to produce toxins. In addition, the Environment Protection Agency (EPA) has included *S. cerevisiae* as a recipient microorganism for exemptions from EPA review and expedited EPA review (40 CFR 725.420). This exemption was made because this species is found to have little adverse effects.

Thus, the RBSC AR Yeast strains belong to the *S. cerevisiae* species, which have been used for more than 5,000 years by humans in bread and fermented beverages. It can therefore be concluded that species will remain GRAS, even after adaptive evolution to constitutively degrade an amino acid for which it has the native cellular machinery.

3.1.2. Source of host strain

The host industrial baker's yeast strain (*S. cerevisiae*) was obtained directly as a commercial culture from a product manufacturer.

3.1.3. Confirmation of taxonomic identification

3.1.3.1. Genetic identification

As a result of its ubiquity and conservation across biological kingdoms, ribosomal DNA (rDNA) is the gold standard for species identification. In fungi, rDNA is organized into a large subunit (25S), small subunit (18S), and an internal transcribed spacer (ITS) region. Although any of these regions can be used to identify species, the ITS region has been established as the universal DNA barcode marker for fungi (67). Moreover, comparing all three regions can substantially increase confidence when making species determinations.

To confirm the identity of the parent industrial baker's yeast strain as *S. cerevisiae*, we sequenced the 18S, 25S, and ITS region of rDNA and compared it to that of the S288C *S. cerevisiae* reference genome. As shown in **Figures 5, 6, and 7**, we observed a perfect match of all three regions in the parent strain to those in S288C, thereby confirming the parent strain's identity as *S. cerevisiae*.

3.1.3.2. Phenotypic identification

Supplementary to rDNA sequencing, a phenotypic analytic profile index (API) can also be used to identify various yeast species. The index uses the presence or absence of growth on 20 different carbon sources to differentiate between species. In this way, the commercially available API platform (Biomérieux) can be used to identify unknown yeast species.

To further confirm the identity of the parent industrial baker's yeast strain as *S. cerevisiae*, we determined its API profile and compared it to that of an *S. cerevisiae* reference strain. As shown in **Figure 8**, API results typed the parent industrial baker's yeast strain as *S. cerevisiae*.

It is recognized that human pathogenic isolates of yeast are able to grow at 42 °C while non-pathogenic strains are not (68). To confirm the non-pathogenic nature of the parent baker's yeast, we tested the growth of the strain at 42 °C compared to its standard temperature of 30 °C. As shown in **Figure 9**, the parent baker's yeast strain does not grow at 42 °C.

3.2. Safety assessment of the evolved strain

3.2.1. Taxonomic identity

Given the confirmed taxonomic identity of the parent baker's yeast strain as *S. cerevisiae* **(Figures 5, 6, 7, and 8)**, we also wanted to confirm the identity of the evolved RBSC AR Yeast. To do so, we compared the 18S, 25S, and ITS region rDNA sequences of the evolved strain to those of the parent, as well as the S288C *S. cerevisiae* reference genome. As shown in **Figures 5, 6, and 7**, we observed a perfect match of all three regions in the evolved strain to those in the parent and S288C, thereby confirming the evolved strain's identity as *S. cerevisiae*.

In addition, we also used API analysis to phenotypically confirm the identity of the evolved RBSC AR Yeast. To do so, we determined the API profile of the AR Yeast and compared it to that of the parent industrial baker's yeast strain. As shown in **Figure 8**, API results typed the evolved AR Yeast strain the same as the parent industrial baker's yeast strain, that is S*. cerevisiae*.

3.2.2. Strain identity

The genome of *S. cerevisiae* contains repetitive DNA sequences (long terminal repeats) known as delta elements. These delta elements are the remnants of Ty1 transposon integration events, and the number and location of these delta elements are specific to a strain. Thus, inter-delta fingerprinting is commonly used to identify and differentiate between strains of *S. cerevisiae* (69, 70).

In order to confirm the parentage of the RBSC AR Yeast strain, inter-delta fingerprinting PCR was used to type the AR Yeast strain against the parent. We observed an identical number, size distribution, and relative intensity of bands, as compared to the parent strain **(Figure 10, compare lanes 3 and 4)**. Importantly, the parent and AR Yeast strains typed differently than the non-related S288C control strains **(Figure 10, lanes 2, 3 and 4)**.

Taken together, we conclude that RBSC AR Yeast strain is derived from the parental industrial baker's yeast S. cerevisiae strain. Moreover, no major DNA reorganization events occurred during classical mutagenesis and adaptive evolution, indicating that the RBSC AR Yeast strain is highly likely to have the same phenotypic properties e.g. safety, growth kinetics, vitality, and suitability for industrial baking use, as its parent.

3.2.3. Growth rate and nutritional requirements

Having used genetic and phenotypic analysis to confirm the identity and parentage of the evolved RBSC AR Yeast **(Figures 5, 6, 7, and 8)**, we next wanted to evaluate the growth rate of the RBSC AR Yeast. Being derived from the parent industrial baker's yeast strain, the RBSC AR Yeast should also grow at the same rate. As shown in **Figure 11**, both the RBSC AR Yeast and its parent strain have similar growth rates. Thus, the classical mutagenesis and adaptive evolution methods used to develop the RBSC AR Yeast did not give the evolved strain any growth advantage, even though the strain has enhanced asparagine catabolic abilities.

A hallmark characteristic of industrial yeast strains is nutritional prototrophy—the ability to synthesize complex metabolites from minimal inorganic nutrients. This is important trait for industrial yeast strains, such that they are able to grow and perform in a wide variety of different nutritional environments. As such, we tested the nutritional prototrophy of the parental baker's yeast strain and the evolved RBSC AR Yeast strain by plating on minimal media (YNB with ammonium sulfate and without amino acids). As shown in **Figure 12**, both the parental baker's yeast strain and the evolved RBSC AR Yeast strain grow efficiently on minimal media, confirming that they are both nutritional prototrophs. In contrast, the laboratory strain BY4741, which is known to be auxotrophic for histidine, leucine, methionine and uracil, did not grow efficiently on the minimal media **(Figure 12)** Moreover, this further establishes the fact that the classical mutagenesis and adaptive evolution methods used to develop the RBSC AR Yeast did not change fundamental properties of the strain, and that RBSC AR Yeast strain is substantially equivalent to the parental strain, except an increased ability to degrade asparagine.

Having shown that the parent baker's yeast strain does not grow at 42 °C and is, thus, non-pathogenic, we wanted to verify that the evolved RBSC AR Yeast also does not grow at 42 °C. As shown in **Figure 9**, the evolved RBSC AR Yeast is incapable of growth at 42 °C, indicating that the classical mutagenesis and adaptive evolution process did not induce pathogenicity traits in the strain.

3.2.4. Classical mutagenesis and adaptive evolution

Classical mutagenesis and adaptive evolution (selection) techniques are well known to the FDA and have been utilized in numerous GRAS notifications to which the agency has no questions (GRN 000103, 000106, 000122, 000142, 000296, 000315, 000333, 000345, 000402, 000406, 000428, and 000507). More specifically, each of these notifications makes use of mutagenized bacterial or yeast strains for the recombinant expression of food-grade enzymes. In each of these notifications, the notifiers rely on the sound assumption that classical mutagenesis techniques do not change the underlying non-pathogenic and non-toxic nature of the microorganism in question (44, 71). Therefore, it should follow that classical mutagenesis of *S. cerevisiae* should not change the safe and accepted use of baker's yeast in food production for humans.

3.3. Safety assessment of the products derived from the evolved strain

3.3.1. Changes in the processing procedures as a consequence of Acrylamide-Reducing Yeast addition

The use of the RBSC AR Yeast leads to no changes in the processes where baker's yeast strains are generally employed (example, baking). In processes that have never incorporated yeast strains, the addition of AR yeast may alter the process and the degree of modification would depend on the food/beverage. Any such modification would not affect the safety of the product but it is recognized that sensory characteristics may be affected if the dose of yeast were at a high concentration.

3.3.2. Reduction of asparagine and acrylamide

In addition to completing experiments using laboratory media containing asparagine, we have also completed simulated food preparation trials using typical ingredients used in the production of grain-based-foods and vegetable-based food products. Under normal food processing conditions we achieved significant reductions in asparagine immediately after the addition of the RBSC AR Yeast. If the food processing method allowed additional contact time prior to heat inactivation, the asparagine levels would dramatically decrease to non-detectable levels, generally within a one to two hour period (depending on dose of yeast, temperature, water activity, type of product etc.)

Hence, the RBSC AR Yeast strain reduces more asparagine than the parental strain, which would result in reducing acrylamide formation in the final food product.

3.3.3. Other undesirable substances

In contrast to recombinant DNA techniques, which offer precision in making genetic modifications, classical mutagenesis and adaptive evolution rely on random mutations to create genetic variability. In this way, classical mutagenesis must be paired with selection/evolution steps in order to identify variants harboring mutations of interest i.e. variants with desired phenotypes. Another important caveat of this approach is that strains displaying desired phenotypes very likely contain mutations other that those responsible for said phenotype. These 'accessory' mutations, which occur proportionally to the amount of mutagenesis/evolution employed, are distributed throughout the genome, including bystander genes.

While accessory mutations have to the potential to be detrimental, this possibility has not deterred the widespread use of classical mutagenesis and selection/evolution in the food industry. As described in Sections 2.2 and 3.2.2, classical mutagenesis of non-pathogenic, non-toxic organisms, which have a history of safe use by humans in food, is accepted as a safe and reliable technique. This view is held both by the food manufacturing industry and by the FDA, as evidenced by the breadth of commercial products employing mutagenesis, and the number of ‘no questions’ GRAS notifications closed by the agency, respectively.

Furthermore, a review of the use of classical mutagenesis in food-grade microorganisms indicates that, in a number of cases, microbes were “subjected to classical mutagenesis and selection to reduce its toxigenic potential” (71). Moreover, in one particular study of the effects of mutagenesis on the yeast *Aspergillus niger*, the authors tested the production of mycotoxins under optimal conditions and showed that the mutagenized strain “showed a pattern of secondary metabolites [e.g. mycotoxins] similar to that of the ancestral strain”, indicating that mutagenesis did not alter *A. niger* toxicity (72).

The safe use of classical mutagenesis and selection/evolution in *S. cerevisiae* (and other related food and beverage *Saccharomyces sp.*) has also been well established. Indeed, classical mutagenesis and selection/evolution have been used to safely modify industrial brewing, winemaking, and baking strains of yeast for properties such as osmotic stress tolerance, freeze tolerance, off-flavor production, alcohol production, alcohol tolerance, and temperature tolerance (44). In all of these cases, no adverse properties were identified in the mutagenized yeast, nor were any risks to human health identified.

4. Conclusion

In this notice Renaissance BioScience Corp. affirms that the use of its acrylamide-reducing baker's yeast to prevent acrylamide formation in food and beverages is GRAS. The acrylamide-reducing yeast is adaptively evolved from a commercial baker's yeast strain such that it constitutively expresses its native *ASP3* gene that encodes a cell wall-associated asparagine-degrading enzyme (Asparaginase II). Recombinant DNA technology was NOT used in the development of this strain and, as such, there is no foreign, or non-native *S. cerevisiae* DNA present in the genome of this strain.

As a basis for this GRAS affirmation, multiple lines of evidence were used to confirm the identify of the acrylamide-reducing yeast as *S. cerevisiae*. Moreover, this evidence also confirms that the acrylamide-reducing yeast is an evolved derivative of its commercial baker's yeast parent, and that the acrylamide-reducing yeast retains the baker's yeast-defining characteristics of its parent. As such, the acrylamide-reducing yeast should be regarded as substantially equivalent to the parent yeast strain in terms of its safety, utility and functionality, with the exception of its ability to degrade asparagine, thereby reducing acrylamide.

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Figures

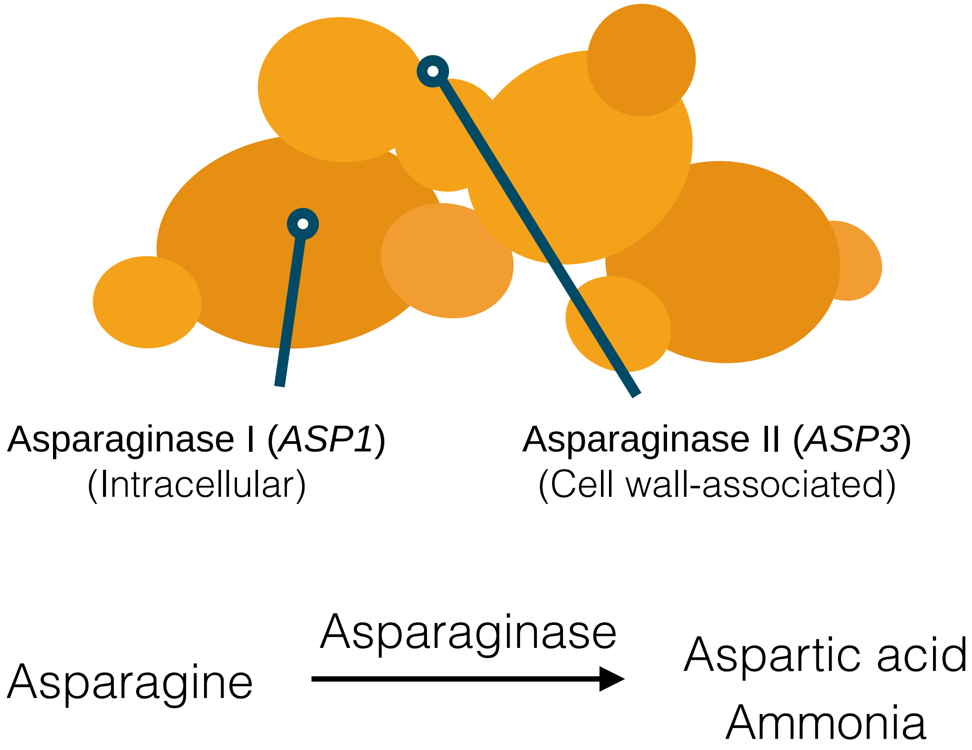

Figure 1 | Schematic representation of asparagine catabolism in *S. cerevisiae.*

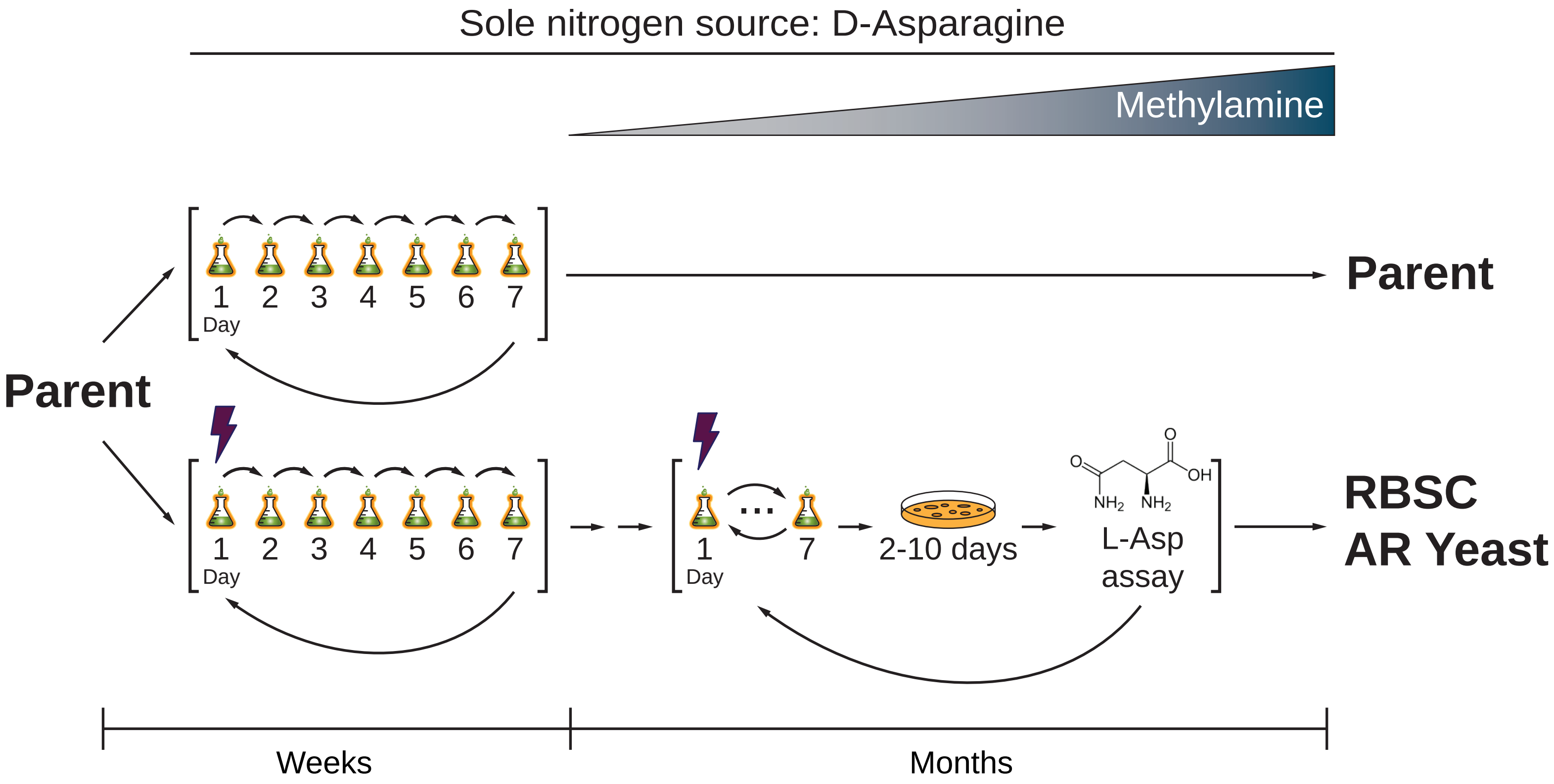

Figure 2 | Schematic representation of the classical mutagenesis and adaptive evolution methodology used to develop the RBSC AR Yeast.

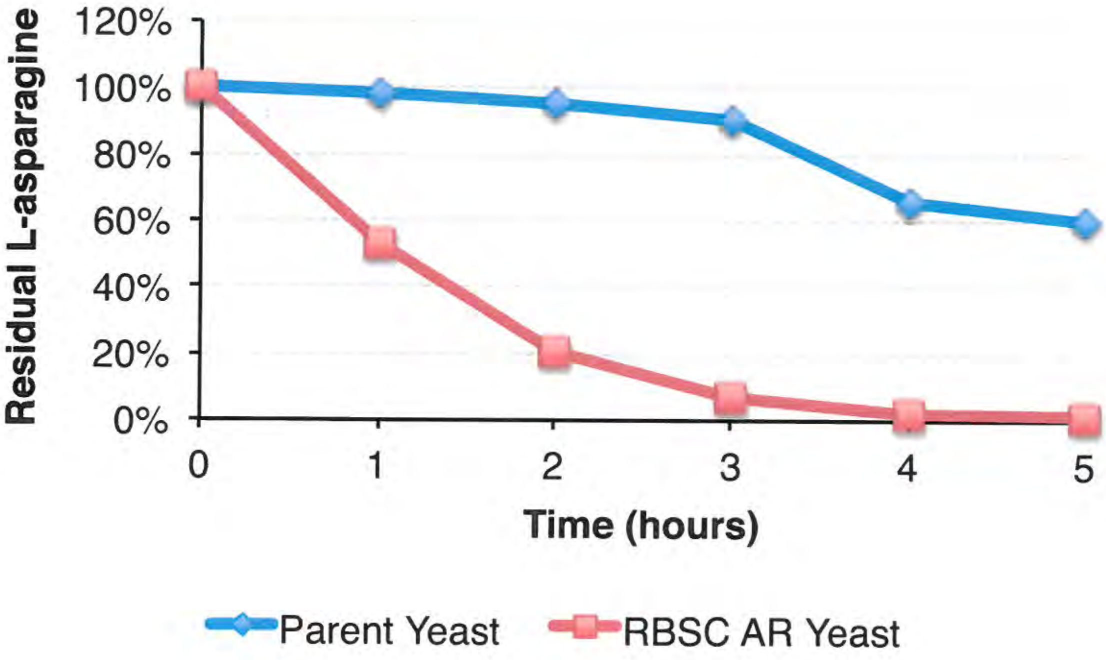

Figure 3 I RBSC AR Yeast strains degrade L-asparagine even when grown in rich media. The parent strain and the RBSC AR Yeast strain were grown in YEG overnight (18 hours at 30 °C) before inoculation of 2 x 107 cells into 5 mL of fresh YEG supplemented with 0.6 g/L L-asparagine. Cells were incubated in YEG + L-asparagine for various time points before heat inactivation at 80 °C. Residual L-asparagine concentration in each sample was measured by colorimetric enzymatic assay kit. Data are representative of duplicate experiments.

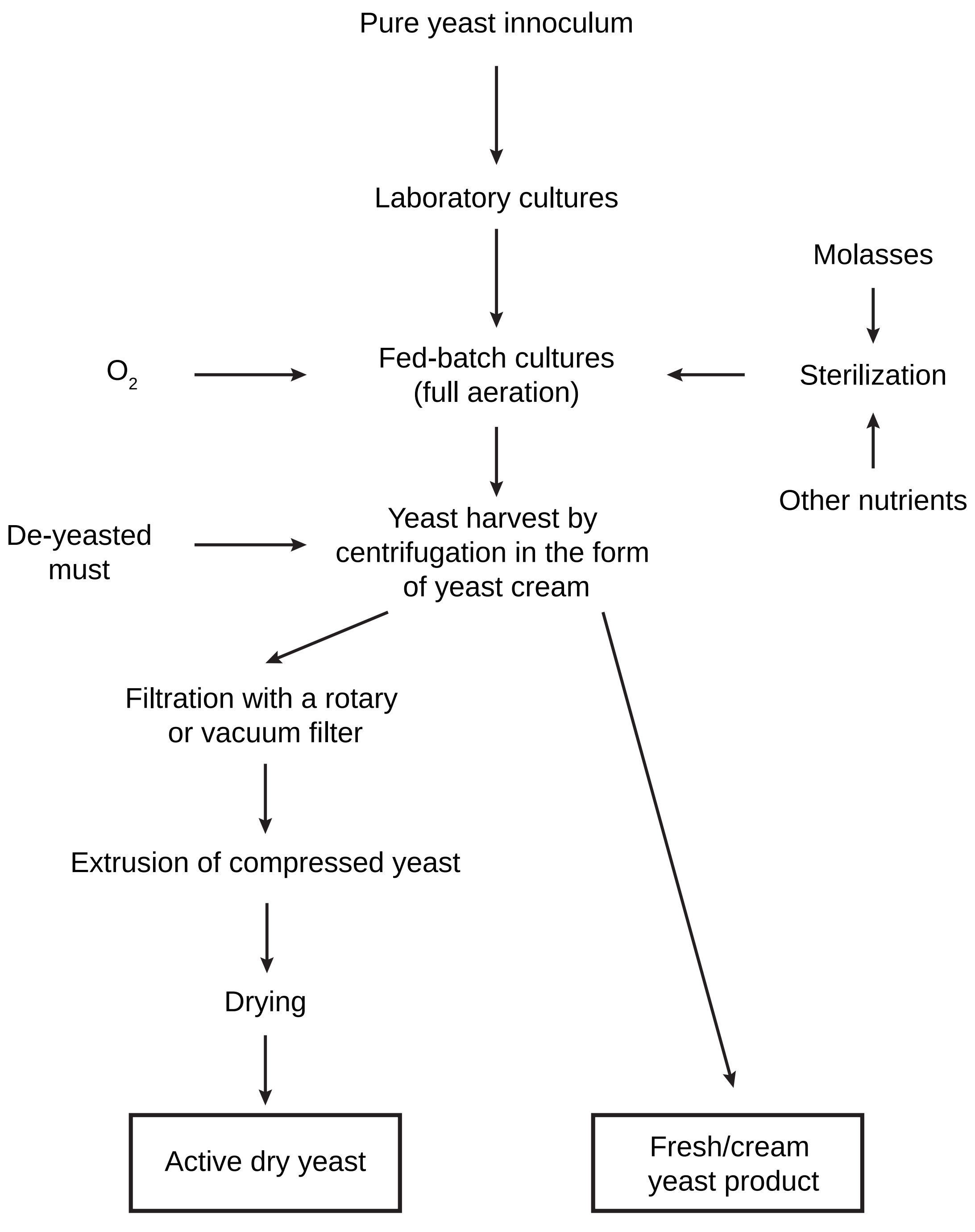

Figure 4 | Schematic representation of the manufacturing process for producing the RBSC AR Yeast in either an active dry or fresh format.

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CLUSTAL O(1.2.1) multiple sequence alignment


S288C_laboratory_strain-ITS      TTTAAGAACATTGTTCGCCTAGACGCTCTCTTCTTATCGATAACGTTCCAATACGCTCAG         60
Parent_yeast_strain-ITS          TTTAAGAACATTGTTCGCCTAGACGCTCTCTTCTTATCGATAACGTTCCAATACGCTCAG         60
Evolved_yeast_strain-ITS         TTTAAGAACATTGTTCGCCTAGACGCTCTCTTCTTATCGATAACGTTCCAATACGCTCAG         60
                                 ************************************************************

S288C_laboratory_strain-ITS      TATAAAAAAAGATTAGCCGCAGTTGGTAAAACCTAAAACGACCGTACTTGCATTATACCT         120
Parent_yeast_strain-ITS          TATAAAAAAAGATTAGCCGCAGTTGGTAAAACCTAAAACGACCGTACTTGCATTATACCT         120
Evolved_yeast_strain-ITS         TATAAAAAAAGATTAGCCGCAGTTGGTAAAACCTAAAACGACCGTACTTGCATTATACCT         120
                                 ************************************************************

S288C_laboratory_strain-ITS      CAAGCACGCAGAGAAACCTCTCTTTGGAAAAAAAACATCCAATGAAAAGGCCAGCAATTT         180
Parent_yeast_strain-ITS          CAAGCACGCAGAGAAACCTCTCTTTGGAAAAAAAACATCCAATGAAAAGGCCAGCAATTT         180
Evolved_yeast_strain-ITS         CAAGCACGCAGAGAAACCTCTCTTTGGAAAAAAAACATCCAATGAAAAGGCCAGCAATTT         180
                                 ************************************************************

S288C_laboratory_strain-ITS      CAAGTTAACTCCAAAGAGTATCACTCACTACCAAACAGAATGTTTGAGAAGGAAATGACG         240
Parent_yeast_strain-ITS          CAAGTTAACTCCAAAGAGTATCACTCACTACCAAACAGAATGTTTGAGAAGGAAATGACG         240
Evolved_yeast_strain-ITS         CAAGTTAACTCCAAAGAGTATCACTCACTACCAAACAGAATGTTTGAGAAGGAAATGACG         240
                                 ************************************************************

S288C_laboratory_strain-ITS      CTCAAACAGGCATGCCCCCTGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATG         300
Parent_yeast_strain-ITS          CTCAAACAGGCATGCCCCCTGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATG         300
Evolved_yeast_strain-ITS         CTCAAACAGGCATGCCCCCTGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATG         300
                                 ************************************************************

S288C_laboratory_strain-ITS      ATTCACGGAATTCTGCAATTCACATTACGTATCGCATTTCGCTGCGTTCTTCATCGATGC         360
Parent_yeast_strain-ITS          ATTCACGGAATTCTGCAATTCACATTACGTATCGCATTTCGCTGCGTTCTTCATCGATGC         360
Evolved_yeast_strain-ITS         ATTCACGGAATTCTGCAATTCACATTACGTATCGCATTTCGCTGCGTTCTTCATCGATGC         360
                                 ************************************************************

S288C_laboratory_strain-ITS      GAGAACCAAGAGATCCGTTGTTGAAAGTTTTTAATATTTTAAAATTTCCAGTTACGAAAA         420
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Evolved_yeast_strain-ITS         GAGAACCAAGAGATCCGTTGTTGAAAGTTTTTAATATTTTAAAATTTCCAGTTACGAAAA         420
                                 ************************************************************

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Parent_yeast_strain-ITS          TTCTTGTTTTTGACAAAAATTTAATGAATAGATAAAATTGTTTGTGTTTGTTACCTCTGG         480
Evolved_yeast_strain-ITS         TTCTTGTTTTTGACAAAAATTTAATGAATAGATAAAATTGTTTGTGTTTGTTACCTCTGG         480
                                 ************************************************************

S288C_laboratory_strain-ITS      GCCCCGATTGCTCGAATGCCCAAAGAAAAAGTTGCAAAGATATGAAAACTCCACAGTGTG         540
Parent_yeast_strain-ITS          GCCCCGATTGCTCGAATGCCCAAAGAAAAAGTTGCAAAGATATGAAAACTCCACAGTGTG         540
Evolved_yeast_strain-ITS         GCCCCGATTGCTCGAATGCCCAAAGAAAAAGTTGCAAAGATATGAAAACTCCACAGTGTG         540
                                 ************************************************************

S288C_laboratory_strain-ITS      TTGTATTGAAACGGTTTTAATTGTCCTATAACAAAAGCACAGAAATCTCTCACCGTTTGG         600
Parent_yeast_strain-ITS          TTGTATTGAAACGGTTTTAATTGTCCTATAACAAAAGCACAGAAATCTCTCACCGTTTGG         600
Evolved_yeast_strain-ITS         TTGTATTGAAACGGTTTTAATTGTCCTATAACAAAAGCACAGAAATCTCTCACCGTTTGG         600
                                 ************************************************************

S288C_laboratory_strain-ITS      AATAGCAAGAAAGAAACTTACAAGCCTAGCAAGACCGCGCACTTAAGCGCAGGCCCGGCT         660
Parent_yeast_strain-ITS          AATAGCAAGAAAGAAACTTACAAGCCTAGCAAGACCGCGCACTTAAGCGCAGGCCCGGCT         660
Evolved_yeast_strain-ITS         AATAGCAAGAAAGAAACTTACAAGCCTAGCAAGACCGCGCACTTAAGCGCAGGCCCGGCT         660
                                 ************************************************************

S288C_laboratory_strain-ITS      GGACTCTCCATCTCTTGTCTTCTTGCCCAGTAAAAGCTCTCATGCTCTTGCCAAAACAAA         720
Parent_yeast_strain-ITS          GGACTCTCCATCTCTTGTCTTCTTGCCCAGTAAAAGCTCTCATGCTCTTGCCAAAACAAA         720
Evolved_yeast_strain-ITS         GGACTCTCCATCTCTTGTCTTCTTGCCCAGTAAAAGCTCTCATGCTCTTGCCAAAACAAA         720
                                 ************************************************************

S288C_laboratory_strain-ITS      AAAATCCATTTTCAAAATTATTAAATTTCTT            751
Parent_yeast_strain-ITS          AAAATCCATTTTCAAAATTATTAAATTTCTT            751
Evolved_yeast_strain-ITS         AAAATCCATTTTCAAAATTATTAAATTTCTT            751
                                 *******************************
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Figure 5 | The Internal Transcribed Spacer (ITS) region (ribosomal DNA) of both the parent yeast strain and the RBSC AR Yeast match that of the prototypical *S. cerevisiae* S288C laboratory strain.
Genomic DNA was harvested from the parent yeast strain and the RBSC AR Yeast strain. DNA sequencing libraries (Nextera XT)—prepared from genomic DNA—were sequenced on an Illumina MiSeq platform in a 2x300 bp paired-end configuration. Raw reads were filtered for quality and mapped to the S288C reference genome.

```
CLUSTAL O(1.2.1) multiple sequence alignment


S288C_laboratory_strain-25S_rDNA      ACAAATCAGACAACAAAGGCTTAATCTCAGCAGATCGTAACAACAAGGCTACTCTACTGC   60
Parent_yeast_strain-25S_rDNA          ACAAATCAGACAACAAAGGCTTAATCTCAGCAGATCGTAACAACAAGGCTACTCTACTGC   60
Evolved_yeast_strain-25S_rDNA         ACAAATCAGACAACAAAGGCTTAATCTCAGCAGATCGTAACAACAAGGCTACTCTACTGC   60
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      TTACAATACCCCGTTGTACATCTAAGTCGTATACAAATGATTTATCCCCACGCAAAATGA   120
Parent_yeast_strain-25S_rDNA          TTACAATACCCCGTTGTACATCTAAGTCGTATACAAATGATTTATCCCCACGCAAAATGA   120
Evolved_yeast_strain-25S_rDNA         TTACAATACCCCGTTGTACATCTAAGTCGTATACAAATGATTTATCCCCACGCAAAATGA   120
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      CATTGCAATTCGCCAGCAAGCACCCAAGGCCTTTCCGCCAAGTGCACCGTTGCTAGCCTG   180
Parent_yeast_strain-25S_rDNA          CATTGCAATTCGCCAGCAAGCACCCAAGGCCTTTCCGCCAAGTGCACCGTTGCTAGCCTG   180
Evolved_yeast_strain-25S_rDNA         CATTGCAATTCGCCAGCAAGCACCCAAGGCCTTTCCGCCAAGTGCACCGTTGCTAGCCTG   180
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      CTATGGTTCAGCGACGCCACAAGGACGCCTTATTCGTATCCATCTATATTGTGTGGAGCA   240
Parent_yeast_strain-25S_rDNA          CTATGGTTCAGCGACGCCACAAGGACGCCTTATTCGTATCCATCTATATTGTGTGGAGCA   240
Evolved_yeast_strain-25S_rDNA         CTATGGTTCAGCGACGCCACAAGGACGCCTTATTCGTATCCATCTATATTGTGTGGAGCA   240
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      AAGAAATCACCGCGTTCTAGCATGGATTCTGACTTAGAGGCGTTCAGCCATAATCCAGCG   300
Parent_yeast_strain-25S_rDNA          AAGAAATCACCGCGTTCTAGCATGGATTCTGACTTAGAGGCGTTCAGCCATAATCCAGCG   300
Evolved_yeast_strain-25S_rDNA         AAGAAATCACCGCGTTCTAGCATGGATTCTGACTTAGAGGCGTTCAGCCATAATCCAGCG   300
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      GATGGTAGCTTCGCGGCAATGCCTGATCAGACAGCCGCAAAAACCAATTATCCGAATGAA   360
Parent_yeast_strain-25S_rDNA          GATGGTAGCTTCGCGGCAATGCCTGATCAGACAGCCGCAAAAACCAATTATCCGAATGAA   360
Evolved_yeast_strain-25S_rDNA         GATGGTAGCTTCGCGGCAATGCCTGATCAGACAGCCGCAAAAACCAATTATCCGAATGAA   360
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      CTGTTCCTCTCGTACTAAGTTCAATTACTATTGCGGTAACATTCATCAGTAGGGTAAAAC   420
Parent_yeast_strain-25S_rDNA          CTGTTCCTCTCGTACTAAGTTCAATTACTATTGCGGTAACATTCATCAGTAGGGTAAAAC   420
Evolved_yeast_strain-25S_rDNA         CTGTTCCTCTCGTACTAAGTTCAATTACTATTGCGGTAACATTCATCAGTAGGGTAAAAC   420
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      TAACCTGTCTCACGACGGTCTAAACCCAGCTCACGTTCCCTATTAGTGGGTGAACAATCC   480
Parent_yeast_strain-25S_rDNA          TAACCTGTCTCACGACGGTCTAAACCCAGCTCACGTTCCCTATTAGTGGGTGAACAATCC   480
Evolved_yeast_strain-25S_rDNA         TAACCTGTCTCACGACGGTCTAAACCCAGCTCACGTTCCCTATTAGTGGGTGAACAATCC   480
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      AACGCTTACCGAATTCTGCTTCGGTATGATAGGAAGAGCCGACATCGAAGAATCAAAAAG   540
Parent_yeast_strain-25S_rDNA          AACGCTTACCGAATTCTGCTTCGGTATGATAGGAAGAGCCGACATCGAAGAATCAAAAAG   540
Evolved_yeast_strain-25S_rDNA         AACGCTTACCGAATTCTGCTTCGGTATGATAGGAAGAGCCGACATCGAAGAATCAAAAAG   540
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      CAATGTCGCTATGAACGCTTGACTGCCACAAGCCAGTTATCCCTGTGGTAACTTTTCTGG   600
Parent_yeast_strain-25S_rDNA          CAATGTCGCTATGAACGCTTGACTGCCACAAGCCAGTTATCCCTGTGGTAACTTTTCTGG   600
Evolved_yeast_strain-25S_rDNA         CAATGTCGCTATGAACGCTTGACTGCCACAAGCCAGTTATCCCTGTGGTAACTTTTCTGG   600
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      CACCTCTAGCCTCAAATTCCGAGGGACTAAAGGATCGATAGGCCACACTTTCATGGTTTG   660
Parent_yeast_strain-25S_rDNA          CACCTCTAGCCTCAAATTCCGAGGGACTAAAGGATCGATAGGCCACACTTTCATGGTTTG   660
Evolved_yeast_strain-25S_rDNA         CACCTCTAGCCTCAAATTCCGAGGGACTAAAGGATCGATAGGCCACACTTTCATGGTTTG   660
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      TATTCACACTGAAAATCAAAATCAAGGGGGCTTTTACCCTTTTGTTCTACTGGAGATTTC   720
Parent_yeast_strain-25S_rDNA          TATTCACACTGAAAATCAAAATCAAGGGGGCTTTTACCCTTTTGTTCTACTGGAGATTTC   720
Evolved_yeast_strain-25S_rDNA         TATTCACACTGAAAATCAAAATCAAGGGGGCTTTTACCCTTTTGTTCTACTGGAGATTTC   720
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      TGTTCTCCATGAGCCCCCCTTAGGACATCTGCGTTATCGTTTAACAGATGTGCCGCCCCA   780
Parent_yeast_strain-25S_rDNA          TGTTCTCCATGAGCCCCCCTTAGGACATCTGCGTTATCGTTTAACAGATGTGCCGCCCCA   780
Evolved_yeast_strain-25S_rDNA         TGTTCTCCATGAGCCCCCCTTAGGACATCTGCGTTATCGTTTAACAGATGTGCCGCCCCA   780
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      GCCAAACTCCCCACCTGACAATGTCTTCAACCCGGATCAGCCCCGAATGGGACCTTGAAT   840
Parent_yeast_strain-25S_rDNA          GCCAAACTCCCCACCTGACAATGTCTTCAACCCGGATCAGCCCCGAATGGGACCTTGAAT   840
Evolved_yeast_strain-25S_rDNA         GCCAAACTCCCCACCTGACAATGTCTTCAACCCGGATCAGCCCCGAATGGGACCTTGAAT   840
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      GCTAGAACGTGGAAAATGAATTCCAGCTCCGCTTCATTGAATAAGTAAAGAAACTATAAA   900
Parent_yeast_strain-25S_rDNA          GCTAGAACGTGGAAAATGAATTCCAGCTCCGCTTCATTGAATAAGTAAAGAAACTATAAA   900
Evolved_yeast_strain-25S_rDNA         GCTAGAACGTGGAAAATGAATTCCAGCTCCGCTTCATTGAATAAGTAAAGAAACTATAAA   900
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      GGTAGTGGTATTTCACTGGCGCCGAAGCTCCCACTTATTCTACACCCTCTATGTCTCTTC   960
Parent_yeast_strain-25S_rDNA          GGTAGTGGTATTTCACTGGCGCCGAAGCTCCCACTTATTCTACACCCTCTATGTCTCTTC   960
Evolved_yeast_strain-25S_rDNA         GGTAGTGGTATTTCACTGGCGCCGAAGCTCCCACTTATTCTACACCCTCTATGTCTCTTC   960
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      ACAATGTCAAACTAGAGTCAAGCTCAACAGGGTCTTCTTTCCCCGCTGATTCTGCCAAGC   1020
Parent_yeast_strain-25S_rDNA          ACAATGTCAAACTAGAGTCAAGCTCAACAGGGTCTTCTTTCCCCGCTGATTCTGCCAAGC   1020
Evolved_yeast_strain-25S_rDNA         ACAATGTCAAACTAGAGTCAAGCTCAACAGGGTCTTCTTTCCCCGCTGATTCTGCCAAGC   1020
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      CCGTTCCCTTGGCTGTGGTTTCGCTAGATAGTAGATAGGGACAGTGGGAATCTCGTTAAT   1080
Parent_yeast_strain-25S_rDNA          CCGTTCCCTTGGCTGTGGTTTCGCTAGATAGTAGATAGGGACAGTGGGAATCTCGTTAAT   1080
Evolved_yeast_strain-25S_rDNA         CCGTTCCCTTGGCTGTGGTTTCGCTAGATAGTAGATAGGGACAGTGGGAATCTCGTTAAT   1080
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      CCATTCATGCGCGTCACTAATTAGATGACGAGGCATTTGGCTACCTTAAGAGAGTCATAG   1140
Parent_yeast_strain-25S_rDNA          CCATTCATGCGCGTCACTAATTAGATGACGAGGCATTTGGCTACCTTAAGAGAGTCATAG   1140
Evolved_yeast_strain-25S_rDNA         CCATTCATGCGCGTCACTAATTAGATGACGAGGCATTTGGCTACCTTAAGAGAGTCATAG   1140
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      TTACTCCCGCCGTTTACCCGCGCTTGGTTGAATTTCTTCACTTTGACATTCAGAGCACTG   1200
Parent_yeast_strain-25S_rDNA          TTACTCCCGCCGTTTACCCGCGCTTGGTTGAATTTCTTCACTTTGACATTCAGAGCACTG   1200
Evolved_yeast_strain-25S_rDNA         TTACTCCCGCCGTTTACCCGCGCTTGGTTGAATTTCTTCACTTTGACATTCAGAGCACTG   1200
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      GGCAGAAATCACATTGCGTCAACATCACTTTCTGACCATCGCAATGCTATGTTTTAATTA   1260
Parent_yeast_strain-25S_rDNA          GGCAGAAATCACATTGCGTCAACATCACTTTCTGACCATCGCAATGCTATGTTTTAATTA   1260
Evolved_yeast_strain-25S_rDNA         GGCAGAAATCACATTGCGTCAACATCACTTTCTGACCATCGCAATGCTATGTTTTAATTA   1260
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      GACAGTCAGATTCCCCTTGTCCGTACCAGTTCTAAGTTGATCGTTAATTGTAGCAAGCGA   1320
Parent_yeast_strain-25S_rDNA          GACAGTCAGATTCCCCTTGTCCGTACCAGTTCTAAGTTGATCGTTAATTGTAGCAAGCGA   1320
Evolved_yeast_strain-25S_rDNA         GACAGTCAGATTCCCCTTGTCCGTACCAGTTCTAAGTTGATCGTTAATTGTAGCAAGCGA   1320
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      CGGTCTACAAGAGACCTACCAAGGCCGTCTACAACAAGGCACGCAAGTAGTCCGCCTAGC   1380
Parent_yeast_strain-25S_rDNA          CGGTCTACAAGAGACCTACCAAGGCCGTCTACAACAAGGCACGCAAGTAGTCCGCCTAGC   1380
Evolved_yeast_strain-25S_rDNA         CGGTCTACAAGAGACCTACCAAGGCCGTCTACAACAAGGCACGCAAGTAGTCCGCCTAGC   1380
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S288C_laboratory_strain-25S_rDNA      AGAGCAAGCCCCACCAAGCAGTCCACAAGCACGCCCGCTGCGTCTGACCAAGGCCCTCAC   1440
Parent_yeast_strain-25S_rDNA          AGAGCAAGCCCCACCAAGCAGTCCACAAGCACGCCCGCTGCGTCTGACCAAGGCCCTCAC   1440
Evolved_yeast_strain-25S_rDNA         AGAGCAAGCCCCACCAAGCAGTCCACAAGCACGCCCGCTGCGTCTGACCAAGGCCCTCAC   1440
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      TACCCGACCCTTAGAGCCAATCCTTATCCCGAAGTTACGGATCTATTTTGCCGACTTCCC   1500
Parent_yeast_strain-25S_rDNA          TACCCGACCCTTAGAGCCAATCCTTATCCCGAAGTTACGGATCTATTTTGCCGACTTCCC   1500
Evolved_yeast_strain-25S_rDNA         TACCCGACCCTTAGAGCCAATCCTTATCCCGAAGTTACGGATCTATTTTGCCGACTTCCC   1500
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      TTATCTACATTATTCTATCAACTAGAGGCTGTTCACCTTGGAGACCTGCTGCGGTTATCA   1560
Parent_yeast_strain-25S_rDNA          TTATCTACATTATTCTATCAACTAGAGGCTGTTCACCTTGGAGACCTGCTGCGGTTATCA   1560
Evolved_yeast_strain-25S_rDNA         TTATCTACATTATTCTATCAACTAGAGGCTGTTCACCTTGGAGACCTGCTGCGGTTATCA   1560
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      GTACGACCTGGCATGAAAACTATTCCTTCCTGTGGATTTTCACGGGCCGTCACAAGCGCA   1620
Parent_yeast_strain-25S_rDNA          GTACGACCTGGCATGAAAACTATTCCTTCCTGTGGATTTTCACGGGCCGTCACAAGCGCA   1620
Evolved_yeast_strain-25S_rDNA         GTACGACCTGGCATGAAAACTATTCCTTCCTGTGGATTTTCACGGGCCGTCACAAGCGCA   1620
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      CCGGAGCCAGCAAAGGTGCTGGCCTCTTCCAGCCATAAGACCCCATCTCCGGATAAACCA   1680
Parent_yeast_strain-25S_rDNA          CCGGAGCCAGCAAAGGTGCTGGCCTCTTCCAGCCATAAGACCCCATCTCCGGATAAACCA   1680
Evolved_yeast_strain-25S_rDNA         CCGGAGCCAGCAAAGGTGCTGGCCTCTTCCAGCCATAAGACCCCATCTCCGGATAAACCA   1680
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      ATTCCGGGGTGATAAGCTGTTAAGAAGAAAAGATAACTCCTCCCAGGGCTCGCGCCGACG   1740
Parent_yeast_strain-25S_rDNA          ATTCCGGGGTGATAAGCTGTTAAGAAGAAAAGATAACTCCTCCCAGGGCTCGCGCCGACG   1740
Evolved_yeast_strain-25S_rDNA         ATTCCGGGGTGATAAGCTGTTAAGAAGAAAAGATAACTCCTCCCAGGGCTCGCGCCGACG   1740
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      TCTCCACATTCAGTTACGTTACCGTGAAGAATCCATATCCAGGTTCCGGAATCTTAACCG   1800
Parent_yeast_strain-25S_rDNA          TCTCCACATTCAGTTACGTTACCGTGAAGAATCCATATCCAGGTTCCGGAATCTTAACCG   1800
Evolved_yeast_strain-25S_rDNA         TCTCCACATTCAGTTACGTTACCGTGAAGAATCCATATCCAGGTTCCGGAATCTTAACCG   1800
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      GATTCCCTTTCGATGGTGGCCTGCATAAAATCAGGCCTTTGAAACGGAGCTTCCCCATCT   1860
Parent_yeast_strain-25S_rDNA          GATTCCCTTTCGATGGTGGCCTGCATAAAATCAGGCCTTTGAAACGGAGCTTCCCCATCT   1860
Evolved_yeast_strain-25S_rDNA         GATTCCCTTTCGATGGTGGCCTGCATAAAATCAGGCCTTTGAAACGGAGCTTCCCCATCT   1860
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      CTTAGGATCGACTAACCCACGTCCAACTGCTGTTGACGTGGAACCTTTCCCCACTTCAGT   1920
Parent_yeast_strain-25S_rDNA          CTTAGGATCGACTAACCCACGTCCAACTGCTGTTGACGTGGAACCTTTCCCCACTTCAGT   1920
Evolved_yeast_strain-25S_rDNA         CTTAGGATCGACTAACCCACGTCCAACTGCTGTTGACGTGGAACCTTTCCCCACTTCAGT   1920
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      CTTCAAAGTTCTCATTTGAATATTTGCTACTACCACCAAGATCTGCACTAGAGGCCGTTC   1980
Parent_yeast_strain-25S_rDNA          CTTCAAAGTTCTCATTTGAATATTTGCTACTACCACCAAGATCTGCACTAGAGGCCGTTC   1980
Evolved_yeast_strain-25S_rDNA         CTTCAAAGTTCTCATTTGAATATTTGCTACTACCACCAAGATCTGCACTAGAGGCCGTTC   1980
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      GACCCGACCTTACGGTCTAGGCTTCGTCACTGACCTCCACGCCTGCCTACTCGTCAGGGC   2040
Parent_yeast_strain-25S_rDNA          GACCCGACCTTACGGTCTAGGCTTCGTCACTGACCTCCACGCCTGCCTACTCGTCAGGGC   2040
Evolved_yeast_strain-25S_rDNA         GACCCGACCTTACGGTCTAGGCTTCGTCACTGACCTCCACGCCTGCCTACTCGTCAGGGC   2040
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      ATCATATCAACCCTGACGGTAGAGTATAGGTAACACGCTTGAGCGCCATCCATTTTCAGG   2100
Parent_yeast_strain-25S_rDNA          ATCATATCAACCCTGACGGTAGAGTATAGGTAACACGCTTGAGCGCCATCCATTTTCAGG   2100
Evolved_yeast_strain-25S_rDNA         ATCATATCAACCCTGACGGTAGAGTATAGGTAACACGCTTGAGCGCCATCCATTTTCAGG   2100
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      GCTAGTTCATTCGGCCGGTGAGTTGTTACACACTCCTTAGCGGATTCCGACTTCCATGGC   2160
Parent_yeast_strain-25S_rDNA          GCTAGTTCATTCGGCCGGTGAGTTGTTACACACTCCTTAGCGGATTCCGACTTCCATGGC   2160
Evolved_yeast_strain-25S_rDNA         GCTAGTTCATTCGGCCGGTGAGTTGTTACACACTCCTTAGCGGATTCCGACTTCCATGGC   2160
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      CACCGTCCGGCTGTCTAGATGAACTAACACCTTTTGTGGTGTCTGATGAGCGTGTATTCC   2220
Parent_yeast_strain-25S_rDNA          CACCGTCCGGCTGTCTAGATGAACTAACACCTTTTGTGGTGTCTGATGAGCGTGTATTCC   2220
Evolved_yeast_strain-25S_rDNA         CACCGTCCGGCTGTCTAGATGAACTAACACCTTTTGTGGTGTCTGATGAGCGTGTATTCC   2220
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      GGCACCTTAACTCTACGTTCGGTTCATCCCGCATCGCCAGTTCTGCTTACCAAAAATGGC   2280
Parent_yeast_strain-25S_rDNA          GGCACCTTAACTCTACGTTCGGTTCATCCCGCATCGCCAGTTCTGCTTACCAAAAATGGC   2280
Evolved_yeast_strain-25S_rDNA         GGCACCTTAACTCTACGTTCGGTTCATCCCGCATCGCCAGTTCTGCTTACCAAAAATGGC   2280
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      CCACTAAAAGCTCTTCATTCAAATGTCCACGTTCAATTAAGTAACAAGGACTTCTTACAT   2340
Parent_yeast_strain-25S_rDNA          CCACTAAAAGCTCTTCATTCAAATGTCCACGTTCAATTAAGTAACAAGGACTTCTTACAT   2340
Evolved_yeast_strain-25S_rDNA         CCACTAAAAGCTCTTCATTCAAATGTCCACGTTCAATTAAGTAACAAGGACTTCTTACAT   2340
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      ATTTAAAGTTTGAGAATAGGTCAAGGTCATTTCGACCCCGGAACCTCTAATCATTCGCTT   2400
Parent_yeast_strain-25S_rDNA          ATTTAAAGTTTGAGAATAGGTCAAGGTCATTTCGACCCCGGAACCTCTAATCATTCGCTT   2400
Evolved_yeast_strain-25S_rDNA         ATTTAAAGTTTGAGAATAGGTCAAGGTCATTTCGACCCCGGAACCTCTAATCATTCGCTT   2400
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      TACCTCATAAAACTGATACGAGCTTCTGCTATCCTGAGGGAAACTTCGGCAGGAACCAGC   2460
Parent_yeast_strain-25S_rDNA          TACCTCATAAAACTGATACGAGCTTCTGCTATCCTGAGGGAAACTTCGGCAGGAACCAGC   2460
Evolved_yeast_strain-25S_rDNA         TACCTCATAAAACTGATACGAGCTTCTGCTATCCTGAGGGAAACTTCGGCAGGAACCAGC   2460
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      TACTAGATGGTTCGATTAGTCTTTCGCCCCTATACCCAAATTCGACGATCGATTTGCACG   2520
Parent_yeast_strain-25S_rDNA          TACTAGATGGTTCGATTAGTCTTTCGCCCCTATACCCAAATTCGACGATCGATTTGCACG   2520
Evolved_yeast_strain-25S_rDNA         TACTAGATGGTTCGATTAGTCTTTCGCCCCTATACCCAAATTCGACGATCGATTTGCACG   2520
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      TCAGAACCGCTACGAGCCTCCACCAGAGTTTCCTCTGGCTTCACCCTATTCAGGCATAGT   2580
Parent_yeast_strain-25S_rDNA          TCAGAACCGCTACGAGCCTCCACCAGAGTTTCCTCTGGCTTCACCCTATTCAGGCATAGT   2580
Evolved_yeast_strain-25S_rDNA         TCAGAACCGCTACGAGCCTCCACCAGAGTTTCCTCTGGCTTCACCCTATTCAGGCATAGT   2580
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      TCACCATCTTTCGGGTCCCAACAGCTATGCTCTTACTCAAATCCATCCGAAGACATCAGG   2640
Parent_yeast_strain-25S_rDNA          TCACCATCTTTCGGGTCCCAACAGCTATGCTCTTACTCAAATCCATCCGAAGACATCAGG   2640
Evolved_yeast_strain-25S_rDNA         TCACCATCTTTCGGGTCCCAACAGCTATGCTCTTACTCAAATCCATCCGAAGACATCAGG   2640
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      ATCGGTCGATTGTGCACCTCTTGCGAGGCCCCAACCTACGTTCACTTTCATTACGCGTAT   2700
Parent_yeast_strain-25S_rDNA          ATCGGTCGATTGTGCACCTCTTGCGAGGCCCCAACCTACGTTCACTTTCATTACGCGTAT   2700
Evolved_yeast_strain-25S_rDNA         ATCGGTCGATTGTGCACCTCTTGCGAGGCCCCAACCTACGTTCACTTTCATTACGCGTAT   2700
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      GGGTTTTACACCCAAACACTCGCATAGACGTTAGACTCCTTGGTCCGTGTTTCAAGACGG   2760
Parent_yeast_strain-25S_rDNA          GGGTTTTACACCCAAACACTCGCATAGACGTTAGACTCCTTGGTCCGTGTTWCAAGACGG   2760
Evolved_yeast_strain-25S_rDNA         GGGTTTTACACCCAAACACTCGCATAGACGTTAGACTCCTTGGTCCGTGTTTCAAGACGG   2760
                                      *************************************************** ********

S288C_laboratory_strain-25S_rDNA      GCGGCATATAACCATTATGCCAGCATCCTTGACTTACGTCGCAGTCCTCAGTCCCAGCTG   2820
Parent_yeast_strain-25S_rDNA          GCGGCATATAACCATTATGCCAGCATCCTTGACTTACGTCGCAGTCCTCAGTCCCAGCTG   2820
Evolved_yeast_strain-25S_rDNA         GCGGCATATAACCATTATGCCAGCATCCTTGACTTACGTCGCAGTCCTCAGTCCCAGCTG   2820
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      GCAGTATTCCCACAGGCTATAATACTTACCGAGGCAAGCTACATTCCTATGGATTTATCC   2880
Parent_yeast_strain-25S_rDNA          GCAGTATTCCCACAGGCTATAATACTTACCGAGGCAAGCTACATTCCTATGGATTTATCC   2880
Evolved_yeast_strain-25S_rDNA         GCAGTATTCCCACAGGCTATAATACTTACCGAGGCAAGCTACATTCCTATGGATTTATCC   2880
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      TGCCACCAAAACTGATGCTGGCCCAGTGAAATGCGAGATTCCCCTACCCACAAGGAGCAG   2940
Parent_yeast_strain-25S_rDNA          TGCCACCAAAACTGATGCTGGCCCAGTGAAATGCGAGATTCCCCTACCCACAAGGAGCAG   2940
Evolved_yeast_strain-25S_rDNA         TGCCACCAAAACTGATGCTGGCCCAGTGAAATGCGAGATTCCCCTACCCACAAGGAGCAG   2940
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      AGGGCACAAAACACCATGTCTGATCAAATGCCCTTCCCTTTCAACAATTTCACGTACTTT   3000
Parent_yeast_strain-25S_rDNA          AGGGCACAAAACACCATGTCTGATCAAATGCCCTTCCCTTTCAACAATTTCACGTACTTT   3000
Evolved_yeast_strain-25S_rDNA         AGGGCACAAAACACCATGTCTGATCAAATGCCCTTCCCTTTCAACAATTTCACGTACTTT   3000
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      TTCACTCTCTTTTCAAAGTTCTTTTCATCTTTCCATCACTGTACTTGTTCGCTATCGGTC   3060
Parent_yeast_strain-25S_rDNA          TTCACTCTCTTTTCAAAGTTCTTTTCATCTTTCCATCACTGTACTTGTTCGCTATCGGTC   3060
Evolved_yeast_strain-25S_rDNA         TTCACTCTCTTTTCAAAGTTCTTTTCATCTTTCCATCACTGTACTTGTTCGCTATCGGTC   3060
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      TCTCGCCAATATTTAGCTTTAGATGGAATTTACCACCCACTTAGAGCTGCATTCCCAAAC   3120
Parent_yeast_strain-25S_rDNA          TCTCGCCAATATTTAGCTTTAGATGGAATTTACCACCCACTTAGAGCTGCATTCCCAAAC   3120
Evolved_yeast_strain-25S_rDNA         TCTCGCCAATATTTAGCTTTAGATGGAATTTACCACCCACTTAGAGCTGCATTCCCAAAC   3120
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      AACTCGACTCTTCGAAGGCACTTTACAAAGAACCGCACTCCTCGCCACACGGGATTCTCA   3180
Parent_yeast_strain-25S_rDNA          AACTCGACTCTTCGAAGGCACTTTACAAAGAACCGCACTCCTCGCCACACGGGATTCTCA   3180
Evolved_yeast_strain-25S_rDNA         AACTCGACTCTTCGAAGGCACTTTACAAAGAACCGCACTCCTCGCCACACGGGATTCTCA   3180
                                      ************************************************************

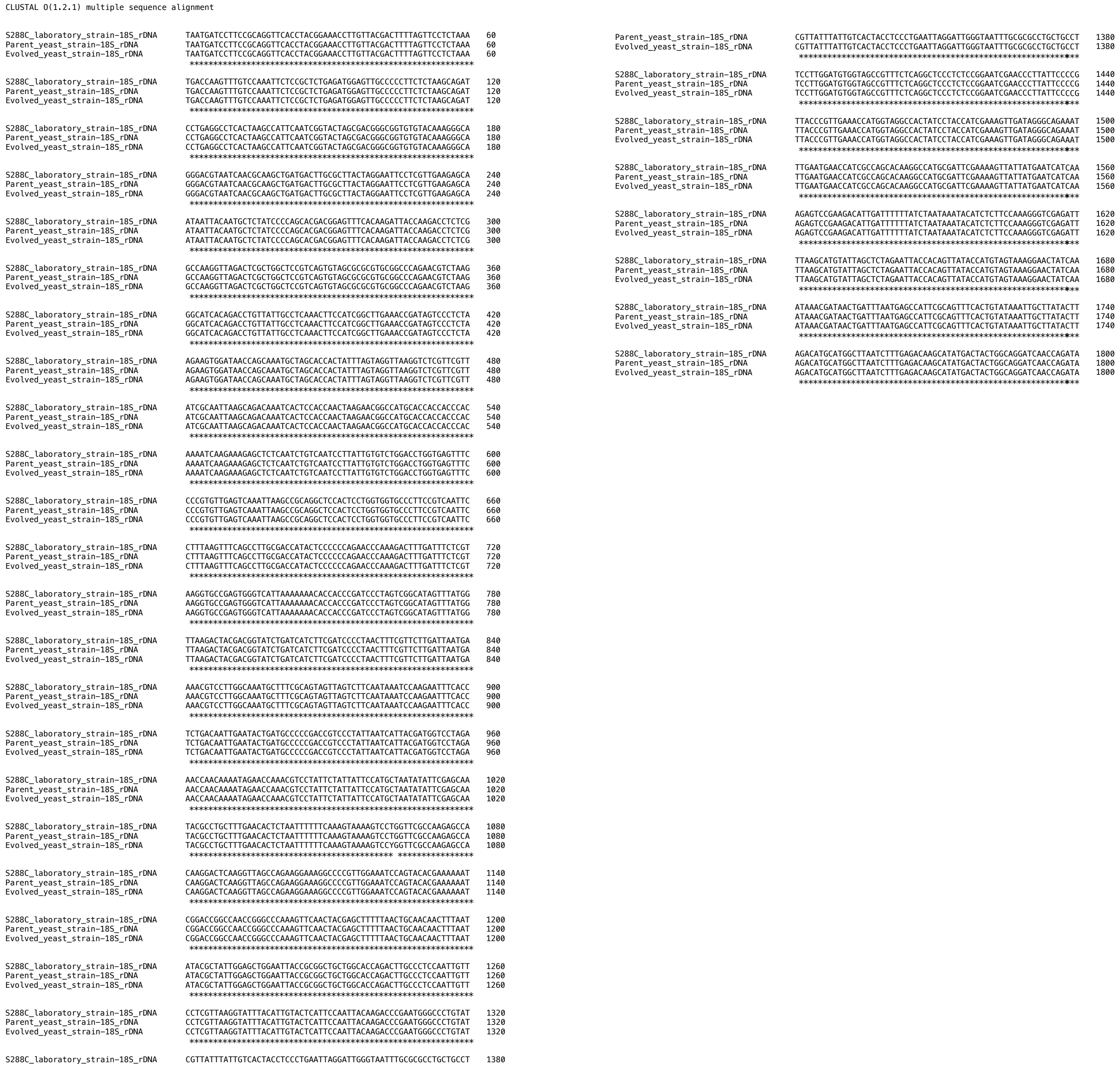
S288C_laboratory_strain-25S_rDNA      CCCTCTATGACGTCCTGTTCCAAGGAACATAGACAAGGAACGGCCCCAAAGTTGCCCTCT   3240
Parent_yeast_strain-25S_rDNA          CCCTCTATGACGTCCTGTTCCAAGGAACATAGACAAGGAACGGCCCCAAAGTTGCCCTCT   3240
Evolved_yeast_strain-25S_rDNA         CCCTCTATGACGTCCTGTTCCAAGGAACATAGACAAGGAACGGCCCCAAAGTTGCCCTCT   3240
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      CCAAATTACAACTCGGGCACCGAAGGTACCAGATTTCAAATTTGAGCTTTTGCCGCTTCA   3300
Parent_yeast_strain-25S_rDNA          CCAAATTACAACTCGGGCACCGAAGGTACCAGATTTCAAATTTGAGCTTTTGCCGCTTCA   3300
Evolved_yeast_strain-25S_rDNA         CCAAATTACAACTCGGGCACCGAAGGTACCAGATTTCAAATTTGAGCTTTTGCCGCTTCA   3300
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      CTCGCCGTTACTAAGGCAATCCCGGTTGGTTTCTTTTCCTCCGCTTATTGATATGCTTAA   3360
Parent_yeast_strain-25S_rDNA          CTCGCCGTTACTAAGGCAATCCCGGTTGGTTTCTTTTCCTCCGCTTATTGATATGCTTAA   3360
Evolved_yeast_strain-25S_rDNA         CTCGCCGTTACTAAGGCAATCCCGGTTGGTTTCTTTTCCTCCGCTTATTGATATGCTTAA   3360
                                      ************************************************************

S288C_laboratory_strain-25S_rDNA      GTTCAGCGGGTACTCCTACCTGATTTGAGGTCAAAC  3396
Parent_yeast_strain-25S_rDNA          GTTCAGCGGGTACTCCTACCTGATTTGAGGTCAAAC  3396
Evolved_yeast_strain-25S_rDNA         GTTCAGCGGGTACTCCTACCTGATTTGAGGTCAAAC  3396
                                      ************************************
```

Figure 6 | The 25S large ribosomal subunit region (ribosomal DNA) of both the parent yeast strain and the RBSC AR Yeast match that of the prototypical *S. cerevisiae* S288C laboratory strain.
Genomic DNA was harvested from the parent yeast strain and the RBSC AR Yeast strain. DNA sequencing libraries (Nextera XT)—prepared from genomic DNA—were sequenced on an Illumina MiSeq platform in a 2x300 bp paired-end configuration. Raw reads were filtered for quality and mapped to the S288C reference genome.



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CLUSTAL O(1.2.1) multiple sequence alignment


S288C_laboratory_strain-18S_rDNA      TAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGACTTTTAGTTCCTCTAAA    60
Parent_yeast_strain-18S_rDNA          TAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGACTTTTAGTTCCTCTAAA    60
Evolved_yeast_strain-18S_rDNA         TAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGACTTTTAGTTCCTCTAAA    60
                                      ************************************************************

S288C_laboratory_strain-18S_rDNA      TGACCAAGTTTGTCCAAATTCTCCGCTCTGAGATGGAGTTGCCCCCTTCTCTAAGCAGAT    120
Parent_yeast_strain-18S_rDNA          TGACCAAGTTTGTCCAAATTCTCCGCTCTGAGATGGAGTTGCCCCCTTCTCTAAGCAGAT    120
Evolved_yeast_strain-18S_rDNA         TGACCAAGTTTGTCCAAATTCTCCGCTCTGAGATGGAGTTGCCCCCTTCTCTAAGCAGAT    120
                                      ************************************************************

S288C_laboratory_strain-18S_rDNA      CCTGAGGCCTCACTAAGCCATTCAATCGGTACTAGCGACGGGCGGTGTGTACAAAGGGCA    180
Parent_yeast_strain-18S_rDNA          CCTGAGGCCTCACTAAGCCATTCAATCGGTACTAGCGACGGGCGGTGTGTACAAAGGGCA    180
Evolved_yeast_strain-18S_rDNA         CCTGAGGCCTCACTAAGCCATTCAATCGGTACTAGCGACGGGCGGTGTGTACAAAGGGCA    180
                                      ************************************************************

S288C_laboratory_strain-18S_rDNA      GGGACGTAATCAACGCAAGCTGATGACTTGCGCTTACTAGGAATTCCTCGTTGAAGAGCA    240
Parent_yeast_strain-18S_rDNA          GGGACGTAATCAACGCAAGCTGATGACTTGCGCTTACTAGGAATTCCTCGTTGAAGAGCA    240
Evolved_yeast_strain-18S_rDNA         GGGACGTAATCAACGCAAGCTGATGACTTGCGCTTACTAGGAATTCCTCGTTGAAGAGCA    240
                                      ************************************************************

S288C_laboratory_strain-18S_rDNA      ATAATTACAATGCTCTATCCCCAGCACGACGGAGTTTCACAAGATTACCAAGACCTCTCG    300
Parent_yeast_strain-18S_rDNA          ATAATTACAATGCTCTATCCCCAGCACGACGGAGTTTCACAAGATTACCAAGACCTCTCG    300
Evolved_yeast_strain-18S_rDNA         ATAATTACAATGCTCTATCCCCAGCACGACGGAGTTTCACAAGATTACCAAGACCTCTCG    300
                                      ************************************************************

S288C_laboratory_strain-18S_rDNA      GCCAAGGTTAGACTCGCTGGCTCCGTCAGTGTAGCGCGCGTGCGGCCCAGAACGTCTAAG    360
Parent_yeast_strain-18S_rDNA          GCCAAGGTTAGACTCGCTGGCTCCGTCAGTGTAGCGCGCGTGCGGCCCAGAACGTCTAAG    360
Evolved_yeast_strain-18S_rDNA         GCCAAGGTTAGACTCGCTGGCTCCGTCAGTGTAGCGCGCGTGCGGCCCAGAACGTCTAAG    360
                                      ************************************************************

S288C_laboratory_strain-18S_rDNA      GGCATCACAGACCTGTTATTGCCTCAAACTTCCATCGGCTTGAAACCGATAGTCCCTCTA    420
Parent_yeast_strain-18S_rDNA          GGCATCACAGACCTGTTATTGCCTCAAACTTCCATCGGCTTGAAACCGATAGTCCCTCTA    420
Evolved_yeast_strain-18S_rDNA         GGCATCACAGACCTGTTATTGCCTCAAACTTCCATCGGCTTGAAACCGATAGTCCCTCTA    420
                                      ************************************************************

S288C_laboratory_strain-18S_rDNA      AGAAGTGGATAACCAGCAAATGCTAGCACCACTATTTAGTAGGTTAAGGTCTCGTTCGTT    480
Parent_yeast_strain-18S_rDNA          AGAAGTGGATAACCAGCAAATGCTAGCACCACTATTTAGTAGGTTAAGGTCTCGTTCGTT    480
Evolved_yeast_strain-18S_rDNA         AGAAGTGGATAACCAGCAAATGCTAGCACCACTATTTAGTAGGTTAAGGTCTCGTTCGTT    480
                                      ************************************************************

S288C_laboratory_strain-18S_rDNA      ATCGCAATTAAGCAGACAAATCACTCCACCAACTAAGAACGGCCATGCACCACCACCCAC    540
Parent_yeast_strain-18S_rDNA          ATCGCAATTAAGCAGACAAATCACTCCACCAACTAAGAACGGCCATGCACCACCACCCAC    540
Evolved_yeast_strain-18S_rDNA         ATCGCAATTAAGCAGACAAATCACTCCACCAACTAAGAACGGCCATGCACCACCACCCAC    540
                                      ************************************************************

S288C_laboratory_strain-18S_rDNA      AAAATCAAGAAAGAGCTCTCAATCTGTCAATCCTTATTGTGTCTGGACCTGGTGAGTTTC    600
Parent_yeast_strain-18S_rDNA          AAAATCAAGAAAGAGCTCTCAATCTGTCAATCCTTATTGTGTCTGGACCTGGTGAGTTTC    600
Evolved_yeast_strain-18S_rDNA         AAAATCAAGAAAGAGCTCTCAATCTGTCAATCCTTATTGTGTCTGGACCTGGTGAGTTTC    600
                                      ************************************************************

S288C_laboratory_strain-18S_rDNA      CCCGTGTTGAGTCAAATTAAGCCGCAGGCTCCACTCCTGGTGGTGCCCTTCCGTCAATTC    660
Parent_yeast_strain-18S_rDNA          CCCGTGTTGAGTCAAATTAAGCCGCAGGCTCCACTCCTGGTGGTGCCCTTCCGTCAATTC    660
Evolved_yeast_strain-18S_rDNA         CCCGTGTTGAGTCAAATTAAGCCGCAGGCTCCACTCCTGGTGGTGCCCTTCCGTCAATTC    660
                                      ************************************************************

S288C_laboratory_strain-18S_rDNA      CTTTAAGTTTCAGCCTTGCGACCATACTCCCCCCAGAACCCAAAGACTTTGATTTCTCGT    720
Parent_yeast_strain-18S_rDNA          CTTTAAGTTTCAGCCTTGCGACCATACTCCCCCCAGAACCCAAAGACTTTGATTTCTCGT    720
Evolved_yeast_strain-18S_rDNA         CTTTAAGTTTCAGCCTTGCGACCATACTCCCCCCAGAACCCAAAGACTTTGATTTCTCGT    720
                                      ************************************************************

S288C_laboratory_strain-18S_rDNA      AAGGTGCCGAGTGGGTCATTAAAAAAACACCACCCGATCCCTAGTCGGCATAGTTTATGG    780
Parent_yeast_strain-18S_rDNA          AAGGTGCCGAGTGGGTCATTAAAAAAACACCACCCGATCCCTAGTCGGCATAGTTTATGG    780
Evolved_yeast_strain-18S_rDNA         AAGGTGCCGAGTGGGTCATTAAAAAAACACCACCCGATCCCTAGTCGGCATAGTTTATGG    780
                                      ************************************************************

S288C_laboratory_strain-18S_rDNA      TTAAGACTACGACGGTATCTGATCATCTTCGATCCCCTAACTTTCGTTCTTGATTAATGA    840
Parent_yeast_strain-18S_rDNA          TTAAGACTACGACGGTATCTGATCATCTTCGATCCCCTAACTTTCGTTCTTGATTAATGA    840
Evolved_yeast_strain-18S_rDNA         TTAAGACTACGACGGTATCTGATCATCTTCGATCCCCTAACTTTCGTTCTTGATTAATGA    840
                                      ************************************************************

S288C_laboratory_strain-18S_rDNA      AAACGTCCTTGGCAAATGCTTTCGCAGTAGTTAGTCTTCAATAAATCCAAGAATTTCACC    900
Parent_yeast_strain-18S_rDNA          AAACGTCCTTGGCAAATGCTTTCGCAGTAGTTAGTCTTCAATAAATCCAAGAATTTCACC    900
Evolved_yeast_strain-18S_rDNA         AAACGTCCTTGGCAAATGCTTTCGCAGTAGTTAGTCTTCAATAAATCCAAGAATTTCACC    900
                                      ************************************************************

S288C_laboratory_strain-18S_rDNA      TCTGACAATTGAATACTGATGCCCCCGACCGTCCCTATTAATCATTACGATGGTCCTAGA    960
Parent_yeast_strain-18S_rDNA          TCTGACAATTGAATACTGATGCCCCCGACCGTCCCTATTAATCATTACGATGGTCCTAGA    960
Evolved_yeast_strain-18S_rDNA         TCTGACAATTGAATACTGATGCCCCCGACCGTCCCTATTAATCATTACGATGGTCCTAGA    960
                                      ************************************************************

S288C_laboratory_strain-18S_rDNA      AACCAACAAAATAGAACCAAACGTCCTATTCTATTATTCCATGCTAATATATTCGAGCAA    1020
Parent_yeast_strain-18S_rDNA          AACCAACAAAATAGAACCAAACGTCCTATTCTATTATTCCATGCTAATATATTCGAGCAA    1020
Evolved_yeast_strain-18S_rDNA         AACCAACAAAATAGAACCAAACGTCCTATTCTATTATTCCATGCTAATATATTCGAGCAA    1020
                                      ************************************************************

S288C_laboratory_strain-18S_rDNA      TACGCCTGCTTTGAACACTCTAATTTTTTCAAAGTAAAAGTCCTGGTTCGCCAAGAGCCA    1080
Parent_yeast_strain-18S_rDNA          TACGCCTGCTTTGAACACTCTAATTTTTTCAAAGTAAAAGTCCTGGTTCGCCAAGAGCCA    1080
Evolved_yeast_strain-18S_rDNA         TACGCCTGCTTTGAACACTCTAATTTTTTCAAAGTAAAAGTCCYGGTTCGCCAAGAGCCA    1080
                                      ******************************************* ****************

S288C_laboratory_strain-18S_rDNA      CAAGGACTCAAGGTTAGCCAGAAGGAAAGGCCCCGTTGGAAATCCAGTACACGAAAAAAT    1140
Parent_yeast_strain-18S_rDNA          CAAGGACTCAAGGTTAGCCAGAAGGAAAGGCCCCGTTGGAAATCCAGTACACGAAAAAAT    1140
Evolved_yeast_strain-18S_rDNA         CAAGGACTCAAGGTTAGCCAGAAGGAAAGGCCCCGTTGGAAATCCAGTACACGAAAAAAT    1140
                                      ************************************************************

S288C_laboratory_strain-18S_rDNA      CGGACCGGCCAACCGGGCCCAAAGTTCAACTACGAGCTTTTTAACTGCAACAACTTTAAT    1200
Parent_yeast_strain-18S_rDNA          CGGACCGGCCAACCGGGCCCAAAGTTCAACTACGAGCTTTTTAACTGCAACAACTTTAAT    1200
Evolved_yeast_strain-18S_rDNA         CGGACCGGCCAACCGGGCCCAAAGTTCAACTACGAGCTTTTTAACTGCAACAACTTTAAT    1200
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S288C_laboratory_strain-18S_rDNA      ATACGCTATTGGAGCTGGAATTACCGCGGCTGCTGGCACCAGACTTGCCCTCCAATTGTT    1260
Parent_yeast_strain-18S_rDNA          ATACGCTATTGGAGCTGGAATTACCGCGGCTGCTGGCACCAGACTTGCCCTCCAATTGTT    1260
Evolved_yeast_strain-18S_rDNA         ATACGCTATTGGAGCTGGAATTACCGCGGCTGCTGGCACCAGACTTGCCCTCCAATTGTT    1260
                                      ************************************************************

S288C_laboratory_strain-18S_rDNA      CCTCGTTAAGGTATTTACATTGTACTCATTCCAATTACAAGACCCGAATGGGCCCTGTAT    1320
Parent_yeast_strain-18S_rDNA          CCTCGTTAAGGTATTTACATTGTACTCATTCCAATTACAAGACCCGAATGGGCCCTGTAT    1320
Evolved_yeast_strain-18S_rDNA         CCTCGTTAAGGTATTTACATTGTACTCATTCCAATTACAAGACCCGAATGGGCCCTGTAT    1320
                                      ************************************************************

S288C_laboratory_strain-18S_rDNA      CGTTATTTATTGTCACTACCTCCCTGAATTAGGATTGGGTAATTTGCGCGCCTGCTGCCT    1380
Parent_yeast_strain-18S_rDNA          CGTTATTTATTGTCACTACCTCCCTGAATTAGGATTGGGTAATTTGCGCGCCTGCTGCCT    1380
Evolved_yeast_strain-18S_rDNA         CGTTATTTATTGTCACTACCTCCCTGAATTAGGATTGGGTAATTTGCGCGCCTGCTGCCT    1380
                                      ************************************************************

S288C_laboratory_strain-18S_rDNA      TCCTTGGATGTGGTAGCCGTTTCTCAGGCTCCCTCTCCGGAATCGAACCCTTATTCCCCG    1440
Parent_yeast_strain-18S_rDNA          TCCTTGGATGTGGTAGCCGTTTCTCAGGCTCCCTCTCCGGAATCGAACCCTTATTCCCCG    1440
Evolved_yeast_strain-18S_rDNA         TCCTTGGATGTGGTAGCCGTTTCTCAGGCTCCCTCTCCGGAATCGAACCCTTATTCCCCG    1440
                                      ************************************************************

S288C_laboratory_strain-18S_rDNA      TTACCCGTTGAAACCATGGTAGGCCACTATCCTACCATCGAAAGTTGATAGGGCAGAAAT    1500
Parent_yeast_strain-18S_rDNA          TTACCCGTTGAAACCATGGTAGGCCACTATCCTACCATCGAAAGTTGATAGGGCAGAAAT    1500
Evolved_yeast_strain-18S_rDNA         TTACCCGTTGAAACCATGGTAGGCCACTATCCTACCATCGAAAGTTGATAGGGCAGAAAT    1500
                                      ************************************************************

S288C_laboratory_strain-18S_rDNA      TTGAATGAACCATCGCCAGCACAAGGCCATGCGATTCGAAAAGTTATTATGAATCATCAA    1560
Parent_yeast_strain-18S_rDNA          TTGAATGAACCATCGCCAGCACAAGGCCATGCGATTCGAAAAGTTATTATGAATCATCAA    1560
Evolved_yeast_strain-18S_rDNA         TTGAATGAACCATCGCCAGCACAAGGCCATGCGATTCGAAAAGTTATTATGAATCATCAA    1560
                                      ************************************************************

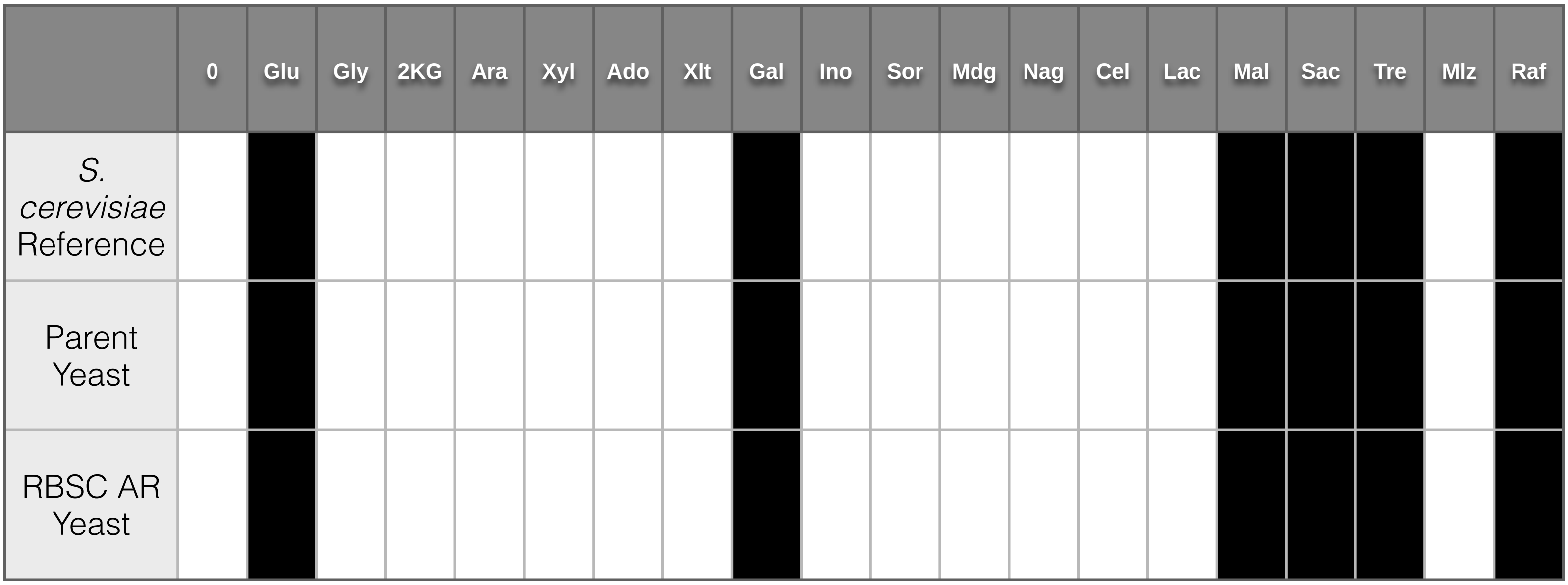
S288C_laboratory_strain-18S_rDNA      AGAGTCCGAAGACATTGATTTTTTATCTAATAAATACATCTCTTCCAAAGGGTCGAGATT    1620
Parent_yeast_strain-18S_rDNA          AGAGTCCGAAGACATTGATTTTTTATCTAATAAATACATCTCTTCCAAAGGGTCGAGATT    1620
Evolved_yeast_strain-18S_rDNA         AGAGTCCGAAGACATTGATTTTTTATCTAATAAATACATCTCTTCCAAAGGGTCGAGATT    1620
                                      ************************************************************

S288C_laboratory_strain-18S_rDNA      TTAAGCATGTATTAGCTCTAGAATTACCACAGTTATACCATGTAGTAAAGGAACTATCAA    1680
Parent_yeast_strain-18S_rDNA          TTAAGCATGTATTAGCTCTAGAATTACCACAGTTATACCATGTAGTAAAGGAACTATCAA    1680
Evolved_yeast_strain-18S_rDNA         TTAAGCATGTATTAGCTCTAGAATTACCACAGTTATACCATGTAGTAAAGGAACTATCAA    1680
                                      ************************************************************

S288C_laboratory_strain-18S_rDNA      ATAAACGATAACTGATTTAATGAGCCATTCGCAGTTTCACTGTATAAATTGCTTATACTT    1740
Parent_yeast_strain-18S_rDNA          ATAAACGATAACTGATTTAATGAGCCATTCGCAGTTTCACTGTATAAATTGCTTATACTT    1740
Evolved_yeast_strain-18S_rDNA         ATAAACGATAACTGATTTAATGAGCCATTCGCAGTTTCACTGTATAAATTGCTTATACTT    1740
                                      ************************************************************

S288C_laboratory_strain-18S_rDNA      AGACATGCATGGCTTAATCTTTGAGACAAGCATATGACTACTGGCAGGATCAACCAGATA    1800
Parent_yeast_strain-18S_rDNA          AGACATGCATGGCTTAATCTTTGAGACAAGCATATGACTACTGGCAGGATCAACCAGATA    1800
Evolved_yeast_strain-18S_rDNA         AGACATGCATGGCTTAATCTTTGAGACAAGCATATGACTACTGGCAGGATCAACCAGATA    1800
                                      ************************************************************
```

Figure 7 | The 18S small ribosomal subunit region (ribosomal DNA) of both the parent yeast strain and the RBSC AR Yeast match that of the prototypical *S. cerevisiae* S288C laboratory strain.
Genomic DNA was harvested from the parent yeast strain and the RBSC AR Yeast strain. DNA sequencing libraries (Nextera XT)—prepared from genomic DNA—were sequenced on an Illumina MiSeq platform in a 2x300 bp paired-end configuration. Raw reads were filtered for quality and mapped to the S288C reference genome.



	0	Glu	Gly	2KG	Ara	Xyl	Ado	Xlt	Gal	Ino	Sor	Mdg	Nag	Cel	Lac	Mal	Sac	Tre	Mlz	Raf
S. cerevisiae Reference	□	■	□	□	□	□	□	□	■	□	□	□	□	□	□	■	■	■	□	■
Parent Yeast	□	■	□	□	□	□	□	□	■	□	□	□	□	□	□	■	■	■	□	■
RBSC AR Yeast	□	■	□	□	□	□	□	□	■	□	□	□	□	□	□	■	■	■	□	■

Figure 8 | The RBSC AR Yeast strain and its parent phenotypically type as *S. cerevisiae.* The RBSC AR Yeast strain, as well as its parent, were typed using the commercially available API 20 C AUX platform (Biomérieux). White cells are negative for growth while black cells indicate positive growth. Data are representative of duplicate experiments.

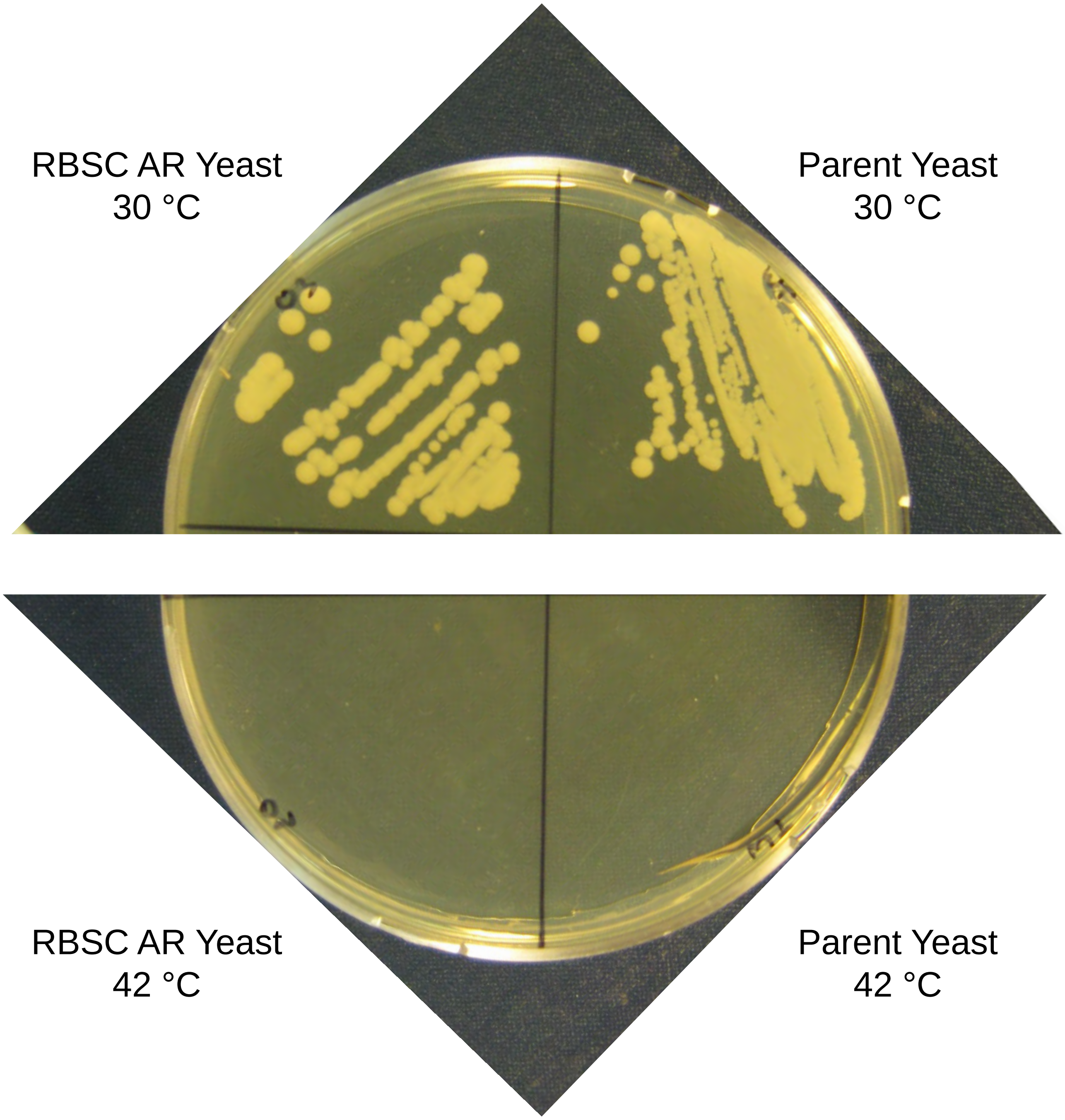


Figure 9 | The RBSC AR Yeast strain and its parent do not grow at 42 °C.
The RBSC AR Yeast strain, as well as its parent, were plated on complete media (YEG: yeast extract + glucose) and grown for three days either at 30 or 42 °C. Data are representative of triplicate experiments.

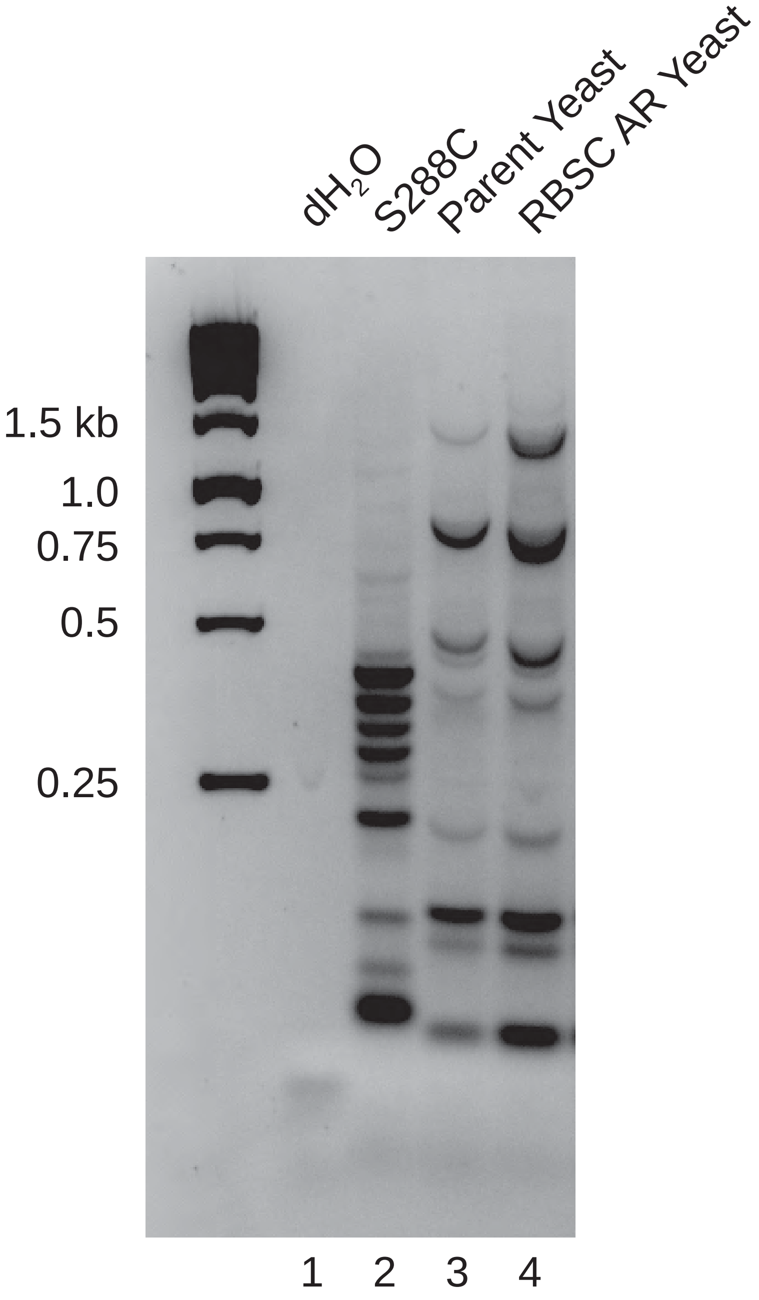


Figure 10 | The RBSC AR Yeast strain is evolutionarily derived from its parent strain. Inter-delta PCR fingerprinting was used to compare the heritage of the RBSC AR Yeast strain to its parent strain. Genomic DNA was extracted from overnight cultures of each of the strains and used as a template for inter-delta PCR. Amplicons were visualized by agarose gel electrophoresis. Data are representative of duplicate experiments.

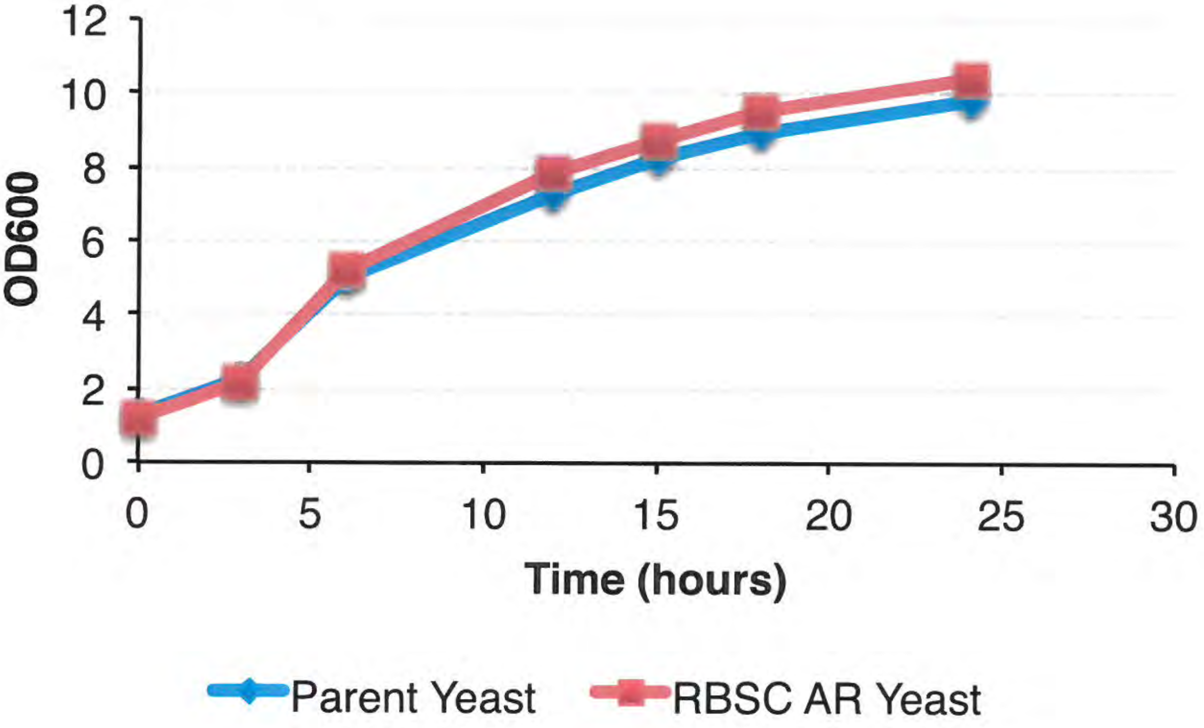


Figure 11 I The RBSC AR Yeast strain and its parent exhibit identical growth rates. The growth rate of the RBSC AR Yeast strain, as well as its parent, were measured by OD_{600} every six hours during growth in YEG. Data are representative of duplicate experiments.

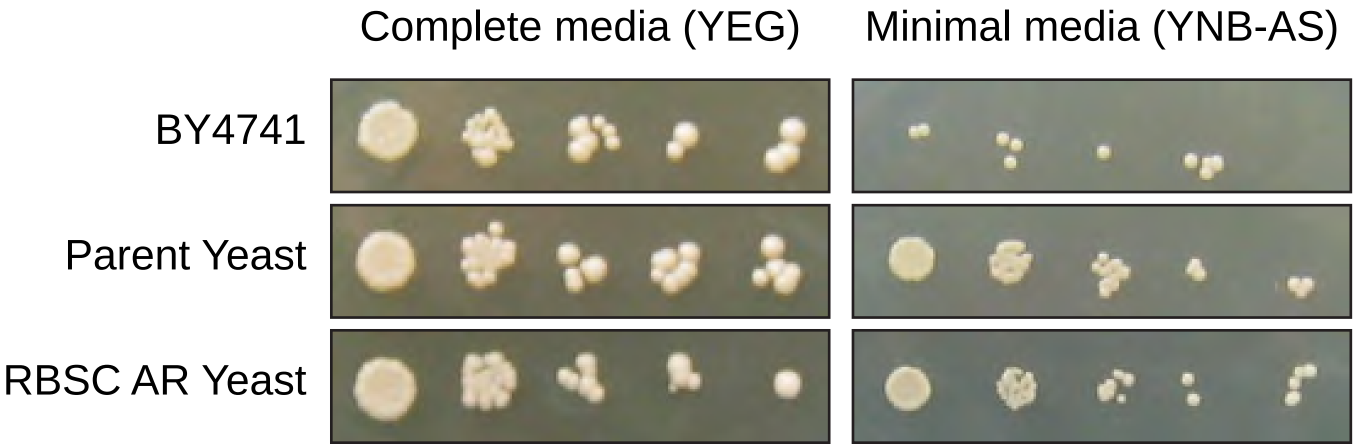


Figure 12 | The RBSC AR Yeast strain and its parent are nutritional prototrophs. The RBSC AR Yeast strain, as well as its parent, were plated in 10 fold serial dilutions on both complete (YEG: yeast extract + glucose) and minimal media (YNB-AS: yeast nitrogen base + glucose + ammonium sulfate) and grown for three days at 30 °C. Data are representative of triplicate experiments.

SUBMISSION END