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Evolutionary engineered *Saccharomyces cerevisiae* wine yeast strains with increased *in vivo* flux through the pentose phosphate pathway

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ABSTRACT

Amplification of the flux toward the pentose phosphate (PP) pathway might be of interest for various *S. cerevisiae* based industrial applications. We report an evolutionary engineering strategy based on a long-term batch culture on gluconate, a substrate that is poorly assimilated by *S. cerevisiae* cells and is metabolized by the PP pathway. After adaptation for various periods of time, we selected strains that had evolved a greater consumption capacity for gluconate. ^{13}C metabolic flux analysis on glucose revealed a redirection of carbon flux from glycolysis towards the PP pathway and a greater synthesis of lipids. The relative flux into the PP pathway was 17% for the evolved strain (ECA5) *versus* 11% for the parental strain (EC1118). During wine fermentation, the evolved strains displayed major metabolic changes, such as lower levels of acetate production, higher fermentation rates and enhanced production of aroma compounds. These represent a combination of novel traits, which are of great interest in the context of modern winemaking.

1. Introduction

The yeast *Saccharomyces cerevisiae* is one of the most widely used industrial organisms. It has been used in baking and in the production of fermented beverages for thousands of years and has more recently become an attractive cell factory for the production of bioethanol and various chemicals.

The pentose phosphate (PP) pathway plays an important role in yeast metabolism, generating NADPH reducing equivalents, together with various precursors for the biosynthesis of nucleic acids and amino acids. This pathway also protects yeast cells against oxidative stress, as NADPH is an essential cofactor for the glutathione- and thioredoxin-dependent enzymes that defend cells against oxidative damage (Izawa et al., 1998; Shenton and Grant, 2003; Slekar et al., 1996).

The importance of this pathway for various biotechnological applications has been emphasized in recent years. For example, recombinant *S. cerevisiae* strains able to ferment biomass-derived pentose sugars have been developed by expressing heterologous xylose reductase and xylitol dehydrogenase genes that metabolize xylose *via* the PP pathway (Jeffries and Jin, 2004). The amount of xylose metabolized and the production levels of ethanol have been increased by modifying the amounts of PP pathway enzymes (Matsushika et al., 2009).

The PP pathway is also an interesting, yet unexplored, target to develop wine yeasts with new properties. Rerouting of carbon flux through the PP pathway may result in a decreased yield of ethanol because one of the carbon atoms of each glucose molecule entering the PP pathway is excreted as CO_2. The development of low-alcohol yeasts is a current challenge to the wine industry since wines produced by modern winemaking practices have excessively high contents of ethanol (Heux et al., 2008). The other effects of an increased flux through the PP pathway on yeast metabolism are predictable. In particular, these include a reduction of acetate formation, due to the major role of both the PP and acetate pathways in the production of NADPH (Grabowska and Chelstowska, 2003; Saint-Prix et al., 2004). Although wine yeasts have been selected for low acetate production, excessive production — which is detrimental for wine quality — is still observed, with some commercial strains having otherwise good technological features. Moreover, winemaking practices, such as grape must clarification usually favor the production of acetic acid (Ribéreau-Gayon et al., 2005).

During the last 15 years, many efforts have been made to engineer wine yeast strains with new characteristics (Dequin, 2001; Donalies et al., 2008; Husnik et al., 2006; Schuller and Casal, 2005). For example, several attempts have been made to divert sugar metabolism towards products other than ethanol (Cambon et al., 2006; Eglinton et al., 2002; Ehsani et al., 2009;

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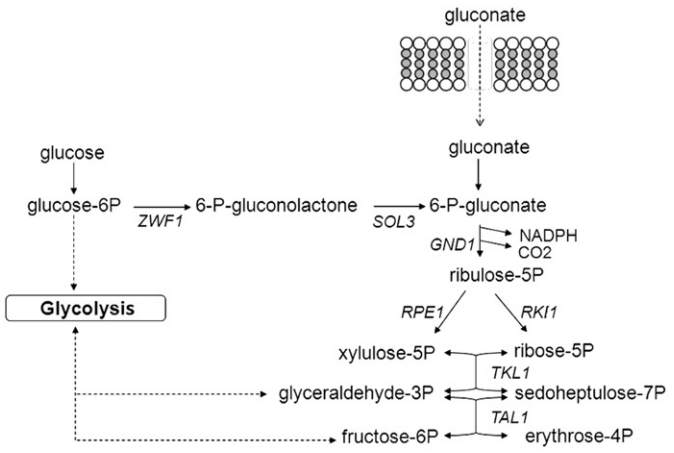

Fig. 1. Gluconate metabolism in *S. cerevisiae.*

Malherbe et al., 2003; Michnick et al., 1997; Nevoigt and Stahl, 1996; Remize et al., 2001; Remize et al., 1999) or to reduce the yield of acetate (Cambon et al., 2006; Remize et al., 2000). However, the use of such approaches is currently limited by poor public acceptance of GMO-based food and beverages. Strategies based on evolutionary engineering are attractive, because they may generate improved strains that in the short-term can be used in industry. Evolution-based approaches have proven valuable — with and without coupling to metabolic engineering — for the generation of new strains with specific phenotypes, such as resistance to various stresses or efficient substrate utilization (Becker and Boles, 2003; Cakar et al., 2009, 2005; Kuyper et al., 2004; Sonderegger and Sauer, 2003; Sonderegger et al., 2004; Wisselink et al., 2007, 2009).

The objective of this study was to design an evolution-based strategy to increase flux through the PP pathway and to evaluate the potential of this strategy during wine fermentation. We developed a strategy based on long-term batch cultivation on δ-gluconolactone, which is metabolized *via* the PP pathway after phosphorylation to generate 6-phosphogluconate (Sinha and Maitra, 1992) (Fig. 1). This hexose sugar is a weak carbon source for *S. cerevisiae* and its assimilation requires oxygen (Gancedo and Delgado, 1984; Sinha and Maitra, 1992). Using this evolutionary engineering strategy, we progressively obtained several strains with improved assimilation of gluconate. These strains displayed various novel phenotypes, including modified adhesion properties, improved fermentation performances, lower levels of acetate production and higher levels of aroma production during wine fermentation. Flux distribution analysis revealed that the evolved ECA5 strain displayed a 1.5 times greater flux through the PP pathway and higher levels of lipid production.

2. Materials and methods

2.1. Yeast strain

The wine yeast strain EC1118 was used as the parental strain. the genome of which has recently been sequenced (Novo et al., 2009). *S. cerevisiae* strains were propagated in rich YPD medium (1% Bacto yeast extract, 2% bactopeptone, 2% glucose).

2.2. Growth conditions

2.2.1. Adaptation experiment

The adaptation procedure was based on a long-term serial transfer procedure. The original strain was taken from a −80 °C stock and spread onto a YPD plate. The biomass from this plate was used to inoculate a tube of 50 ml containing 20 ml of YPD broth. After an overnight culture at 28 °C, we used the resulting cell suspension to inoculate two capped tubes (13 ml), each containing 5 ml of SD gluconate medium (0.67% nitrogen base, 2% gluconate) pH 6 (adjusted with a solution of NaOH 32%) to an OD_{600} of 0.1. The tubes were then incubated at 28 °C, with shaking (250 rpm). When the OD_{600} reached 0.8, fresh medium was inoculated to an OD_{600} of 0.1. The population was tested for assimilation of gluconate at regular intervals. Clones isolated from adaptation cultures using a streak-plate isolation procedure and exhibiting the same properties as the total adapted population were selected as evolved strains and stored at −80 °C in 40% glycerol.

2.2.2. Determination of growth parameters

We incubated 50 ml aerobic batch cultures in 250 ml flasks on a rotary shaker (300 rpm), at 28 °C. The medium used was minimal SD medium containing 6.7 g l^{-1} yeast nitrogen base without amino acids (Difco) and 20 g l^{-1} of glucose (pH 5.4) or gluconate (pH 6.5). The pH of the synthetic medium was adjusted to 6.5 when gluconate was used as the carbon source by addition of 32% NaOH solution. The medium was inoculated to an OD_{600} of 0.1, from an overnight culture in 25 ml YPD medium in 50 ml Falcon tubes. The growth rate was determined in an aerobic batch culture, by log-linear regression analyses of OD_{600} against time, with growth rate (μmax) as the regression coefficient. Doubling times (G) were calculated using the following equation: $G = \ln 2/\mu max$.

2.2.3. Oxygenation

Aerobic fermentations were performed in 300 ml fermentors containing 270 ml of SD gluconate, at 28 °C. Fermentors were supplied with 6 and 30 mg l^{-1} of oxygen and agitation was maintained by gentle continuous magnetic stirring at 500 rpm. Dissolved oxygen was measured by an oxygen probe (Clark oxygen probe type YSI5331; Gilson; Middleton, WI, USA). Anaerobic cultures were performed in serum tubing vials of 125 ml containing 100 ml of medium and hermetically closed. Anaerobiosis was obtained by bubbling argon in the medium for 15 min at 95 °C and was monitored *via* the addition of 2 mg l^{-1} resazurin.

2.2.4. Wine fermentation conditions

Batch fermentation experiments were carried out in synthetic medium (MS), which mimics standard grape juice and has been described previously (Bely and Sablayrolles, 1990). The MS medium used in this study contained 240 g l^{-1} glucose, 6 g l^{-1} malic acid, 6 g l^{-1} citric acid, and a nitrogen source composed of 120 mg l^{-1} nitrogen from ammonium chloride and 340 mg l^{-1} nitrogen from amino acids. The following amino acids were used: tyrosine (final concentration 18.5 mg l^{-1}), tryptophane (179 mg l^{-1}), isoleucine (32.7 mg l^{-1}) aspartate (44.5 mg l^{-1}), glutamate (123 mg l^{-1}), arginine (375 mg l^{-1}), leucine (48.5 mg l^{-1}), threonine (76 mg l^{-1}), glycine (18.5 mg l^{-1}), glutamine (505 mg l^{-1}), alanine (145.5 mg l^{-1}), valine (44.5 mg l^{-1}), methionine (31.5 mg l^{-1}), phenylalanine (38 mg l^{-1}), serine (78.5 mg l^{-1}), histidine (32 mg l^{-1}), lysine (17 mg l^{-1}), cysteine (13 mg l^{-1}), and proline (613 mg l^{-1}).

To satisfy the lipid requirements of yeast cells during anaerobic growth, MS medium was supplemented with 7.5 mg l^{-1} ergosterol, 0.21 g l^{-1} Tween 80, and 2.5 mg l^{-1} oleic acid. The pH of the resulting medium was 3.3. We inoculated 250 ml flasks containing 50 ml YPD to an OD_{600} of 0.1 with cells grown overnight in YPD and incubated them for 8 h at 28 or 18 °C. The fermentation culture in MS medium was inoculated with 0.5×10^6 cells per ml and incubated at 28 °C with continuous stirring (350 rpm) in fermentors

(NH verre) with a working volume of 1.0 l equipped with a fermentor condenser. CO_2 release was determined by automatic measurements of fermentor weight every 20 min. The rate of CO_2 production, dCO_2/dt, is the first derivative of the amount of CO_2 produced over time and was calculated automatically by polynomial smoothing of the CO_2 production curve (Sablayrolles et al., 1987). Fermentation experiments were performed in triplicate.

2.3. Analytical methods

Cell numbers were determined by measuring optical density at 600 nm (OD_{600}) or in a Coulter ZB2 cell counter linked to a C56 Channelyzer fitted with a probe with a 100-μm aperture (Beckman Coulter, UK). Dry weights were determined by filtering 10 ml of the culture through pre-weighed nitrocellulose filters (pore size 0.45 μm, Millipore). The filters were washed with 100 ml distilled water and dried until no further change in weight was observed. Glucose, glycerol, ethanol, pyruvate, succinate, acetate, α-ketoglutarate, and 2-hydroxyglutarate levels were analyzed by high-pressure liquid chromatography (HPLC), with an HPX-87H ion exclusion column (Bio-Rad). Volatile aromatic compounds were determined by gas chromatography (GC).

2.4. Adhesion-associated phenotypes

Flocculation (cell–cell adhesion) was assessed by culturing yeast cells in 13 ml tubes containing 5 ml of YPD for 48 h. Cells were washed twice with 10 mM EDTA buffer and twice with sterile water. Flocculation was induced by adding 5 ml of flocculation buffer (final concentrations: 3 mM of calcium chloride and 50 mM of sodium acetate). The tubes were shaken at 50 rpm for 5 min and flocculation was estimated, by eye, under the microscope.

Invasive growth was determined in a plate-washing assay, as previously described (Roberts and Fink, 1994). Cells were dispensed onto YPD plates, incubated for 3 days at 28 °C and washed. Plates were photographed before and after washing. The density of residual cells on the plate (agar adhesion intensity) was determined with IRIS software (version 5.57).

We assayed adhesion to plastic in a polystyrene 96-well microtiter plate, using a method described by Reynolds and Fink (2001), with minor modifications. Cells were grown in YPD overnight, washed and resuspended in SD glucose medium, to an OD_{600} of 1. The resulting cell suspension (0.1 ml) was transferred to a microtiter plate and incubated for 24 h at 28 °C. After washing, the attached yeast cells were stained by incubation for 30 min with 150 μl of 1% (w/v) crystal violet in H_2O. The microtiter plates were washed with water and photographed. The excess crystal violet was then removed in 150 μl of absolute ethanol. We determined the OD_{570} for each well with a Powerwave 340 (Biotek, Winooski, VT). The score for adhesion to plastic was defined as the intensity of crystal violet staining. Each strain was tested six times and the experiment was performed in duplicate.

2.5. Carbon labeling and metabolic network analysis

We grew 10 ml batch cultures in serum tubing vials of 12.5 ml filled with MS medium containing 100 g l^{-1} of a mixture of 40% ^{13}C labeled on C1 (Eurisotop) and 60% unlabeled glucose and 14 mg l^{-1} of NH_4Cl as the sole nitrogen source. Cells were harvested at an OD_{600} of 3, which corresponded to the mid-exponential phase.

We carried out acid hydrolysis of the biomass for the determination of amino acids and labeling patterns. One glucose derivative, glucose pentacetate, and two different amino-acid derivatives, ethyl chloroformate (ECF), and dimethyl formamide dimethyl acetal (DMFDMA), were analyzed by GC–MS (Christensen and Nielsen, 1999), as previously described (Gombert et al., 2001). From the raw data obtained by GC–MS, the summed fractional labeling (SFL) of each fragment was calculated using

$$SFL = 100 \times [(1m_1 + 2m_2 + \ldots + nm_n) \times (m_0 + m_1 + m_2 + \ldots + m_n)^{-1}],$$

where m_0 is the fractional abundance of the lowest corrected mass and $m_{i>0}$ is the abundance of molecules with higher corrected masses. We calculated 25 SFLs and measured 22 fluxes, including 15 for biomass formation and 7 for metabolites, giving a total of 47 measurements.

The metabolic network consisted of 60 reactions. Flux calculations were carried out with Matlab 7, with the slightly modified mathematical framework, as previously described (Christensen and Nielsen, 1999; Gombert et al., 2001). The differences between experimental and simulated SFLs and between experimental and simulated fluxes were minimized by an iterative procedure. The calculation routine was performed 100 times and the fluxes reported are the means of the convergent solutions.

2.6. Analysis of cellular components and composition

Intracellular carbohydrate content was determined as previously described (Albersheim et al., 1967; Harris et al., 1984). Proteins were extracted from cells by incubation with 50% (vol/vol) DMSO for one hour at 60 °C and quantified with a BC Assay kit (Uptima-Interchim, France). Proteins were hydrolyzed by incubation for 24 h at 120 °C in 6 N HCl, and amino-acid composition was then determined by HPLC, as previously described (Pripis-Nicolau et al., 2000). Lipid content was determined by weighing after extraction with chloroform/methanol (2/1 (vol/vol)), as previously described (Bligh and Dyer, 1959).

2.7. Principal component analysis

Patterns within the different sets of data were investigated by principal component analysis (PCA). PCA is a bilinear statistical modeling method for decreasing the number of variables and identifying relationships between variables. We used centered data and R Development Core Team software, with the specific package ade4 (Dray, 2007), for this analysis. Hierarchical cluster analysis was performed on scaled parameters, with the Euclidean metric and the Ward method used to analyze linkage clustering in the FactoMineR package (Dray, 2007).

3. Results

3.1. Assimilation of gluconate by S. cerevisiae

δ-Gluconolactone has been described as a substrate, which is poorly assimilated by *S. cerevisiae*. Its utilization is facilitated by a culture pH higher than 3.8 (to favor the gluconate form) and by aerobic conditions (Barnett and Payne, 1990; Sinha and Maitra, 1992). We first investigated the ability of the EC1118 strain to grow aerobically on plates containing 2% gluconate as the sole carbon source. This strain could grow on this substrate, but formed considerably less biomass than on glucose, and only when the pH exceeded 4, confirming the utilization of gluconate rather than δ-gluconolactone. We evaluated the oxygen requirements of the EC1118 strain by culturing this strain in SD medium containing 2% gluconate under anaerobic conditions or in the presence of oxygen dissolved at concentrations of 6 and 30 mg l^{-1}. We showed that oxygen was strictly required for growth. Yeast cells

degraded 1 and 3.8 g l^{-1} gluconate in respective media containing 6 and 30 mg l^{-1} oxygen. Under these conditions, we detected no significant levels of excreted ethanol or the other usual by-products of fermentation, consistent with a metabolic bottleneck in gluconate assimilation. Consistent with this finding, we identified two intermediates of the PP pathway, 6-phosphogluconate and ribose 1,5-bisphosphate in the culture supernatant after increasing concentration by a factor of 200 (data not shown).

3.2. Sequential batch culture on gluconate and selection of evolved strains

EC1118 variants with a greater capacity to grow on gluconate were selected by aerobic batch cultures in SD medium containing 2% gluconate at pH 6, by a serial transfer-dilution strategy. Two parallel evolution experiments were conducted to evaluate the reproducibility of the evolution strategy. Culture aliquots were periodically collected and growth improvement on gluconate was monitored on SD medium containing 2% gluconate by measuring gluconate degradation and population size (OD600). No modification was observed during the 20 first transfers. After 23, 37, and 79 transfers, corresponding to about 70, 180, and 240 generations, a flocculation was observed simultaneously in the two independent cultures. Strains ECA2 and ECA5 (70 generations), ECB2 and ECB5 (180 generations), and ECC2 and ECC5 (240 generations) were collected at these different time points (series 2 and 5 correspond to independently propagated cultures) and were further characterized for growth on gluconate and glucose.

Gluconate consumption and cell number progressively increased during the evolution experiment (Fig. 2). These two traits evolved in a similar manner in both of the independently propagated cultures. The evolved strains generated populations which were 1.2–4.6 times larger than that of EC1118 strain, and consumed 1.5–9 times as much gluconate (Fig. 2), with no effect on the growth rate in this medium (Fig. 2). These data suggest that growth improvement on gluconate may result from mutations on enzymes involved in the gluconate assimilation pathway. However, compared with the EC1118 strain, the rate of gluconate consumption was not increased for the evolved strains, suggesting that some bottlenecks in the gluconate assimilation pathway have not yet been removed.

All of the evolved strains were able to consume 2% glucose during culture in SD medium. The growth rate on glucose was similar for both evolved and EC1118 strains, but the population of evolved strains gradually decreased on this carbon source (Fig. 2).

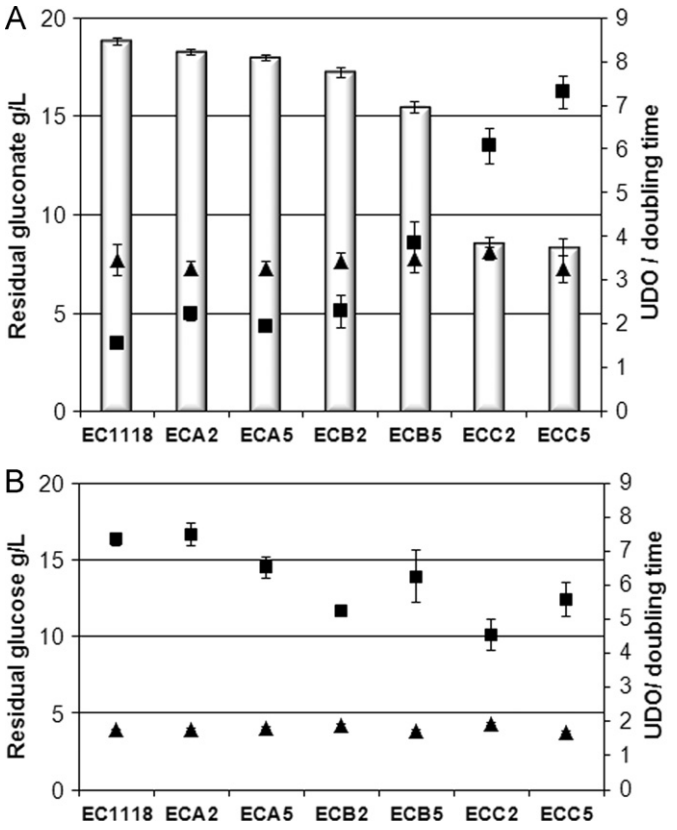

Fig. 2. Growth and substrate consumption of the evolved strains after aerobic growth on 2% gluconate (A) and on 2% glucose (B). The final biomass (black squares) and doubling time (h) (black triangles) on gluconate or glucose were determined. The final amount of gluconate is represented by histogram. Glucose was completely consumed.

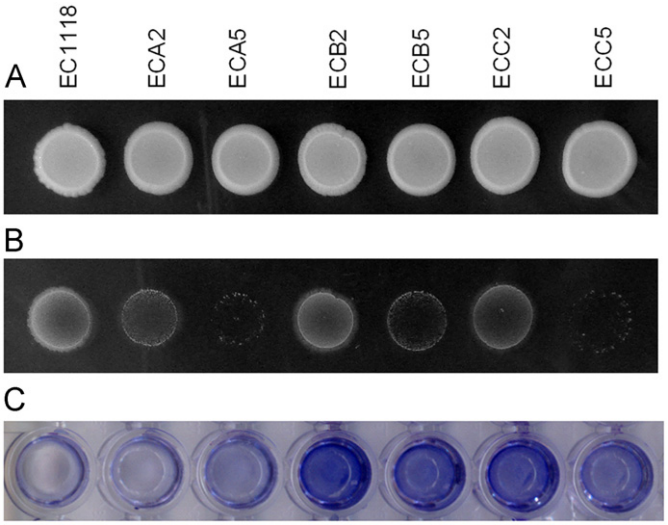

Fig. 3. Invasive growth and adhesion to plastic of parental and evolved strains. (A, B) *Invasive growth* Yeast strains were streaked onto agar plates and incubated for 3 days at 28 °C. Photographs were taken (A) after incubation (prewash) and (B) directly after intense washing under a stream of water (postwash). (C) *Adhesion to plastic* Strains were grown in 96-well plates for 24 h, in SD medium. Cells were stained with crystal violet and washed. Plastic adhesion is observed as a cell layer remaining attached to the plate after washing.

3.3. Adherence properties

Further characterization of the properties of cells sampled from the evolution experiment revealed that they exhibited Ca^{2+}-dependent flocculation and that they formed pseudohyphae, visible under the microscope. The simultaneous occurrence of these two phenotypes suggests that the cell may display a coordinated response to nutrient deprivation (Fichtner et al., 2007). The flocculines implicated in pseudohyphal growth and flocculation are also involved in adherence to solid surfaces and invasive growth. To better understand this phenomenon, we therefore investigated whether the overall adhesion properties of the evolved strains had been modified on glucose.

The EC1118 strain is non-flocculent and can grow invasively but cannot adhere to plastic (Fig. 3). Like the parental strain, the evolved strains did not flocculate (data not shown). Strains ECA2, ECB2, ECC2 retained the ability to grow invasively, but this trait was lost in the strains from series 5 (Fig. 3B). In contrast, all of the evolved strains adhered more strongly to plastic than to the EC1118 strain. Adhesion capacity was progressively increased by the adaptation strategy, peaking after 180 generations (Fig. 3C).

These data suggest that the flocculation observed during the evolution experiment is due to a transient response to nutrient deprivation, whereas other adhesion properties might result from stable genetic modification. Invasive growth and plastic adhesion are mediated by *FLO11*, which contains in-frame coding sequence repeats whose variations in copy number have been shown to modify adhesion characteristics and have been implicated in evolutionary processes (Fidalgo et al., 2006; Fidalgo et al., 2008). Therefore, the differences in adhesion properties between independently propagated cultures may result from variations in the number of *FLO11* repeat sequences during the evolution experiment.

3.4. Behavior of the evolved strains during wine fermentation

We studied the behavior of the evolved strains during wine fermentation on synthetic medium (Dickinson and Williams, 1986), simulating the composition of grape must. All strains except for ECB5 fermented the synthetic must more rapidly than the parental strain, as shown by the higher maximal fermentation rate and the shorter duration of fermentation (Table 1). This result was all the more remarkable given that they also formed less biomass than EC1118 strain. The evolved strains produced between 1.6 and 2.6 less times less acetate than EC1118 strain (Table 1). The production of other by-products, including ethanol, was unaffected compared with the EC1118 strain.

In contrast, there were significant changes in the levels of volatile aroma compounds. Ester levels were markedly higher. The production of ethyl acetate, isoamyl acetate, and 2-phenyl acetate all increased during adaptation, reaching a maximum in the ECB2 and ECB5 strains and decreasing thereafter, in the ECC2 and ECC5 strains. The maximal levels of isoamyl acetate and 2-phenyl acetate were, respectively, 25 and 15-fold higher than those for the EC1118 strain. The production of higher levels of alcohols (2-phenylethanol, isobutanol, and isoamyl alcohol) followed the same trend as the production of esters.

To better characterize the fermentative properties of the evolved strains, we performed fermentations on MS medium containing 100 mg l^{-1} of assimilable nitrogen (MS70) at a low temperature (18 °C). Under these conditions, the ECA5 strain exhibited the better fermentative properties, as shown by obtaining complete sugar degradation 200 hours earlier than EC1118 strain (Fig. 4). The other evolved strains exhibited fermentative properties that were intermediary between the EC1118 and ECA5 strains (ECA2, ECB5, ECC5), or did not consume all of the glucose (ECB2 and ECC2) (data not shown).

The evolved ECA5 strain grew better than the parental strain on gluconate but also continued to perform well on glucose, with a different metabolic profile and better fermentation kinetics during wine fermentation than the parental yeast EC1118 (Table 1, Fig. 4). We therefore decided to investigate this strain further.

3.5. Comparative ^{13}C flux analysis for ECA5 and EC1118 strains

We studied the metabolic characteristics of this strain by carrying out a comparative ^{13}C flux analysis for the ECA5 and

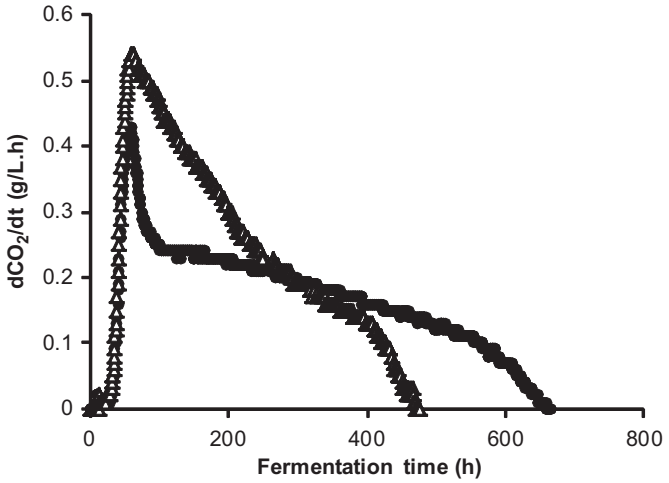

Fig. 4. Fermentation performances (dCO_2/dt) of ECA5 strain (white triangle) in comparison with EC1118 strain (black circle) on MS70 medium at 18 °C.

Table 1
Metabolite and biomass levels and fermentation parameters of EC1118 and evolved strains during wine fermentation (MS medium containing 240 g/l glucose). Values shown are the average of three independent experiments ± standard deviation.

	EC1118	ECA2	ECA5	ECB2	ECB5	ECC2	ECC5
Main compounds (g l^{-1})							
Glucose	240.3 ± 3.5	240.3 ± 3.5	240.3 ± 3.5	240.3 ± 3.5	240.3 ± 3.5	240.3 ± 3.5	240.3 ± 3.5
Biomass	4.7 ± 0.1	4.5 ± 0.1	4.6 ± 0.1	4.1 ± 0.1	4.2 ± 0.1	3.7 ± 0.1	3.7 ± 0.1
Ethanol	112.8 ± 1.6	112.4 ± 2.0	111.8 ± 0.8	113.0 ± 0.1	114.7 ± 0.2	111.8 ± 0.8	111.3 ± 0.9
Glycerol	7.5 ± 0.0	7.2 ± 0.1	8.0 ± 0.2	7.8 ± 0.0	7.0 ± 0.5	7.4 ± 0.5	7.3 ± 0.0
Pyruvate	0.07 ± 0.00	0.07 ± 0.00	0.10 ± 0.00	0.05 ± 0.00	0.10 ± 0.00	0.06 ± 0.00	0.10 ± 0.00
Acetate	0.8 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
Succinate	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	0.5 ± 0.0
Volatile compounds (mg l^{-1})							
Phenyl ethanol	57.1 ± 4.0	80.1 ± 0.3	65.2 ± 0.8	76.5 ± 7.1	91.2 ± 4.5	57.9 ± 1.2	55.1 ± 3.9
Isoamyl alcohol	173.8 ± 0.7	165.2 ± 1.6	166.0 ± 1.6	277.7 ± 14.0	241.8 ± 11. 0	274.3 ± 0.5	163.0 ± 0.7
Isobutanol	28.5 ± 1.7	45.2 ± 0.4	57.9 ± 0.4	91.9 ± 0.9	83.6 ± 0.8	87.1 ± 1.1	32.2 ± 0.5
Phenylethyl acetate	0.2 ± 0.0	1.7 ± 0.0	1.6 ± 0.0	3.1 ± 0.3	3.0 ± 0.3	2.8 ± 0.1	2.7 ± 0.0
Isoamyl acetate	0.5 ± 0.0	3.8 ± 0.3	4.3 ± 0.3	12.5 ± 0.9	11.8 ± 0.8	12.7 ± 0.2	5.4 ± 0.1
Ethyl acetate	38.2 ± 1.7	38.3 ± 2.7	55.4 ± 0.8	55.9 ± 0.7	56.0 ± 5.9	57.8 ± 1.2	58.1 ± 1.5
Ethyl butyrate	0.10 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.09 ± 0.01	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.00
Redox and carbon balances (%)							
Carbon recovery	99	99	99	99	100	98	98
Redox balance	97	97	97	98	98	96	96
Kinetic parameters							
Vmax (gCO_2 l^{-1} h^{-1})	2.8 ± 0.1	2.8 ± 0.1	3.3 ± 0.1	3.2 ± 0.1	3.2 ± 0.1	3.1 ± 0.1	3 ± 0.1
T80 (h)	66 ± 2	59 ± 2	52 ± 1	59 ± 2	61 ± 2	60 ± 2	60 ± 2
Fermentation time (h)	97 ± 2	95 ± 2	80 ± 1	88 ± 2	115 ± 2	93 ± 2	93 ± 2

EC1118 strains. In these two strains, the composition of key biomass constituents (carbohydrates, lipids, proteins, DNA, RNA) was determined experimentally.

Analysis of the flux distributions of the two strains showed substantial differences in flux partitioning between the glycolysis and PP pathways (Fig. 5). For the parental yeast, EC1118, $11 \pm 0.8\%$ of the carbon flux was channeled into the PP pathway, *versus* $17.0 \pm 0.8\%$ for the ECA5 strain. The rerouting of carbon from glycolysis to the PP pathway in the ECA5 strain is consistent with the evolutionary strategy based on adaptation to the use of gluconate — a PP pathway intermediate — as the sole carbon source. The ECA5 strain produced slightly less ethanol than the EC1118 strain (3.4 mM/100 mM glucose, corresponding to 6.8 mmol of carbon (Cmmol)/100 mmol glucose), as expected based on an additional excretion of 6.9 mmol of carbon as CO_2 by the evolved strain.

Flux distribution for pyruvate was typical of fermentative metabolism, with pyruvate being mostly channeled towards ethanol and acetate in both strains (Frick and Wittmann, 2005). However, although the parental and evolved strains had similar levels of acetaldehyde dehydrogenase activity, the flux through the acetyl-CoA synthase reaction was three times higher in ECA5 than in EC1118. Thus, 70% of the acetate produced by the acetaldehyde dehydrogenase reaction was excreted into the medium by the mutant strain, *versus* 90% of that produced by the parental strain. A greater part of acetate produced by ECA5 was used for the formation of acetyl-CoA and lipid synthesis and the lipid content was four times higher in ECA5 than in EC1118 (data not shown).

During glucose fermentation, the TCA pathway operates as two branches with relatively low levels of activity, to provide precursors for anabolism. The parental and evolved strains differed significantly in terms of TCA pathway replenishment with mitochondrial pyruvate and oxaloacetate. The flux for pyruvate import into mitochondria, which meets 50% of the demand for mitochondrial pyruvate in the EC1118 strain, was strongly limited in the ECA5 strain. This strain displayed much higher levels of pyruvate carboxylase and malic enzyme activity than the EC1118 strain. This re-orientation of fluxes around the pyruvate node indicates that, in the evolved strain, the biosynthetic requirements for mitochondrial pyruvate and oxaloacetate are supplied exclusively by anaplerosis.

3.6. Principal component analysis

PCA was used to integrate the various phenotypic traits of the evolved strains obtained. We included the variables that differed

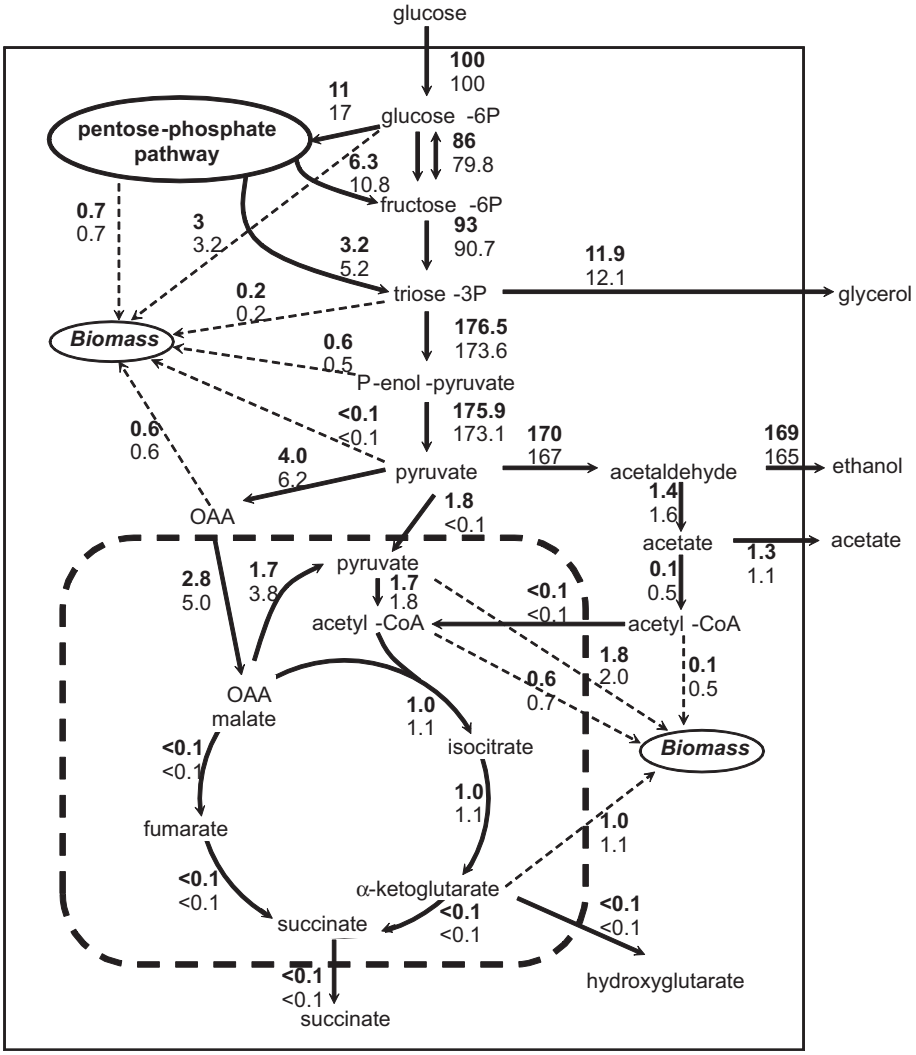

Fig. 5. Intracellular carbon flux distribution of *S. cerevisiae* strains EC1118 (bold) and ECA5 (italics). All fluxes are normalized to the uptake of 100 mmol glucose. For reversible reactions, an additional arrow indicates the net direction of fluxes.

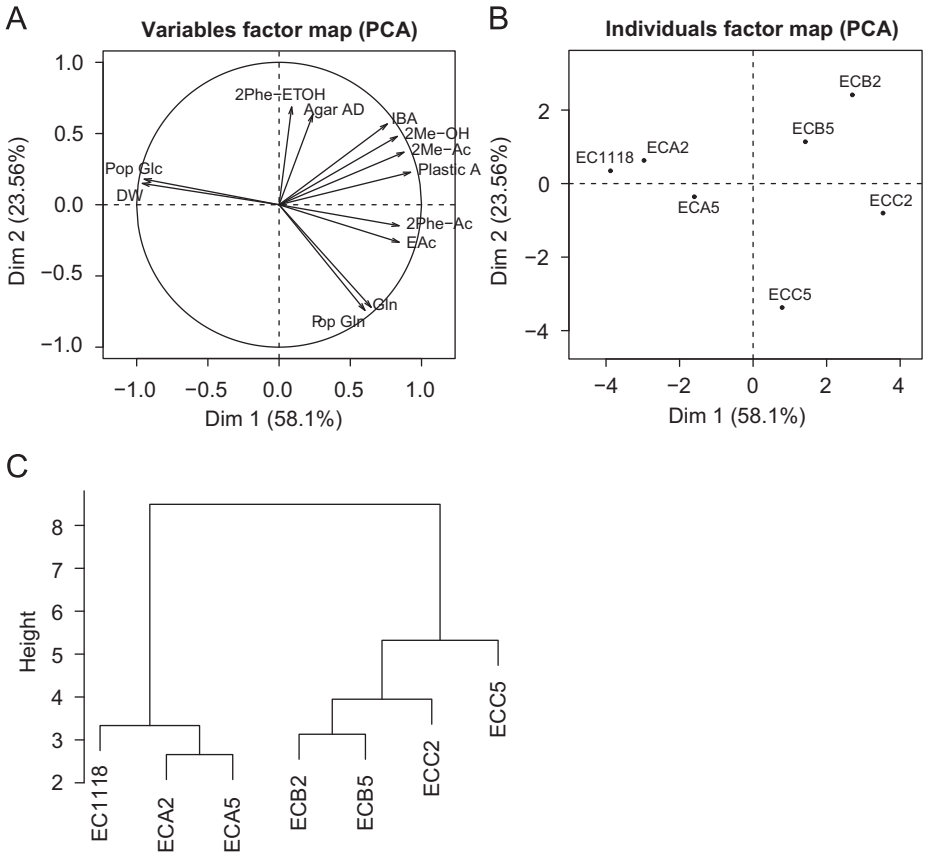

Fig. 6. PCA analysis and hierarchical cluster analysis. (A) Map of variable factors. The variables used for PCA analysis were 2-phenylacetate (2Phe-Ac), isoamyl alcohol (2Me-OH), isoamyl acetate (2ME-Ac), 2-phenylethanol (2Phe-ETOH), ethyl acetate (Eac) and isobutyl acetate (IBA) levels, agar adhesion (Agar AD), plastic adhesion (Plastic AD), final population in SD gluconate medium (Pop Gln), gluconate consumed (Gln), final population in SD glucose medium (Pop Glc) biomass (dry weight) in MS (DW). (B) Distribution of strains on PCA and (C) hierarchical cluster analysis.

most between the strains, namely: 2-phenylacetate, 2-phenylethanol, isoamyl alcohol, isoamyl acetate, isobutanol and ethyl acetate levels, agar adhesion, plastic adhesion, biomass on gluconate, gluconate consumed, maximal population on SD glucose, and final biomass in MS glucose medium. In the resulting PCA plot, the data points are separated along a curve running across the first two principal components, which account for 81.7% of the variance (Fig. 6A). The Dim1 axis (58.1% of the variance) mostly corresponds to the 2-phenylacetate and ethyl acetate variables, on the one hand, and population and biomass on glucose on the other hand; these two groups of variables being negatively correlated ($r=-0.75$) (Fig. 6A).

The levels of all aroma compounds, except 2-phenylethanol, were significantly correlated (r between 0.80 and 0.89). We also found a strong correlation between isoamyl alcohol or isoamyl acetate production and plastic adhesion ($r=0.76$ and 0.89, respectively). There was also a trend for growth parameters on glucose and on gluconate being negatively correlated (r between -0.65 and -0.76).

Fig. 6B shows the projection of the strains in the two-dimensional space defined by the Dim1 and Dim2 axis. The groups of strains were separated in a pattern based on time of sampling. Strains were clearly separated on the basis of their ability to use glucose or gluconate along the first axis, which can be interpreted as substrate specialization. EC1118, ECA2, and ECA5 strains utilized glucose very efficiently, whereas the other strains displayed specialization for gluconate.

On the second axis, groups were separated as a function of aroma production. The ECB2, ECB5, and ECC2 strains produced larger amounts of aroma compounds and adhered to plastic more strongly. Adhesion to plastic was strongly correlated with the production of aroma compounds (Figs. 3 and 6). Fusel alcohols have been shown to induce filamentous growth (Dickinson, 1996). Indeed, *S. cerevisiae* cells release aromatic alcohols as quorum-sensing molecules capable of inducing the morphogenetic switch in response to nutrient starvation (Chen and Fink, 2006). The adhesion properties of the evolved strains might therefore result from an increase in the production of higher alcohols.

We carried out a hierarchical cluster analysis to identify groups of strains with similar characteristics (Fig. 6C). We identified two groups, one containing the first two evolved strains, ECA2 and ECA5, clustered close together and with the parental strain and the second containing ECB2 and ECC5. The structure of this second group suggested a drift between ECC2 and ECC5, reflecting divergence between the independently propagated cultures.

4. Discussion

In this study, we used an evolutionary engineering approach based on long-term culture on gluconate, to select wine yeast strains with enhanced flux through the PP pathway. This strategy proved to be valuable for the selection of strains with greater growth capacity on gluconate. Comparative ^{13}C flux analysis of an evolved strain with the EC1118 strain showed the flux through the PP pathway to be 1.5 times higher in the evolved strain.

The evolved strains selected after 70, 180, and 240 generations exhibited an increase of biomass on SD gluconate and slightly reduced biomass formation on SD glucose (Fig. 2), indicating

a gradual specialization of the selected strains for growth on gluconate. A similar loss of ability to grow on arabinose after the long-term selection of *S. cerevisiae* on xylose has been previously reported (Wisselink et al., 2009).

Detailed characterization of the evolved strains on glucose revealed novel phenotypic traits that were common to all strains (lower levels of biomass formation, higher rates of fermentation, lower levels of acetate production, higher levels of aroma production). PCA analysis of the various phenotypic traits (Fig. 6A and B) showed that the phenotype of the propagated strains had progressively diverged from the ancestral strain. The hierarchical cluster analysis (Fig. 6C) highlighted a progressive divergence between strains from parallel evolution experiments. Long-term molecular evolution experiments with *Escherichia coli* have shown that different random mutations are fixed in different populations and this subtly alters the course of evolution (Woods et al., 2006).

Further characterization of the evolved strains during wine fermentation revealed a number of phenotypic traits, which are potentially of great interest for winemaking. Comparative flux analysis showed that the flux through the PP pathway was 1.5 times greater in the evolved strain ECA5 strain than in EC1118 strain (17% *versus* 11%). This represents a marked increase, of the same magnitude as that obtained when comparing the flux through the PP pathway between a fermentative metabolism (18%) and a respirofermentative metabolism (11%) (Jouhten et al., 2008). The flux analysis provides evidence that rerouting carbon flux through the PP pathway could actually reduce the availability of carbons for ethanol, since the observed decrease in the flux towards ethanol exactly matched the additional excretion of CO_2. However, such a decrease is expected to reduce the ethanol in wine of about 2 g l^{-1}, which is hardly detectable.

Surprisingly, biomass composition measurements and flux analysis revealed that ECA5 produced four times as much lipid as EC1118 strain. As the fatty acid synthesis consumes NADPH, an increase in lipid production in the evolved strain might partly compensate for the NADPH surplus generated by the increase in flux through the PP pathway. In support of this hypothesis, Dickinson and Hewlins showed that a mutant deleted for *ZWF1*, which encodes for the first step of the PP pathway, produced smaller amounts of fatty acids.

The higher flux through the PP pathway of ECA5 strain compared with the EC1118 strain may account for the higher levels of production of 2-phenylethanol and 2-phenylethylacetate, both of which are degradation products of phenylalanine, an amino acid produced in the PP pathway. Other esters produced in larger amounts in the evolved strain (isoamyl acetate, ethyl acetate) are produced from the acyl-CoA derivatives of fatty acids. The increase in their production may result from enhanced flux through the acetyl-CoA and lipid pathway (Fig. 5). Other indirect effects of the strong metabolic reprogramming of the evolved strains, such as changes in amino-acid synthesis, may also be involved and may account for the general increase in esters and higher alcohol levels. These volatile metabolites produced by yeast metabolism are minor but sensorially important (Herrero et al., 2008; Swiegers and Pretorius, 2005). The evolved strains obtained in this study produced higher amounts of flavors generally considered positive in wine, such as 2 phenylethyl alcohol and 2 phenylethyl acetate, associated with a rose-like odor, and isoamyl acetate, associated with a pear and banana aroma.

One other commercially relevant property of the evolved strains was a low production of acetate, which is a particularly desirable attribute for commercial wine yeast strains. During wine fermentation, acetate is produced mostly by the NADPH acetaldehyde dehydrogenase Ald6p (Saint-Prix et al., 2004). A decrease in acetate production in the evolved strains might therefore partially compensate for the surplus NADPH generated by the increase in flux through the PP pathway. However, ^{13}C flux analysis showed that the lower level of acetate excretion by the ECA5 strain, rather than compensating for NADPH overproduction by the PP pathway, may instead result from an increase in carbon flux from acetate towards acetyl-CoA and lipid synthesis.

This study shows that evolutionary strategies can lead to a strong reshaping of central metabolism and paves the way to a generation of strains with novel properties that can quickly be used in the wine industry. A main challenge for the future will be to decipher the processes underlying the phenotypes obtained. In the near future, rapid advances in genome sequencing and in various "omics" technologies should make it possible to determine the genomic basis of complex phenotypes (Bro and Nielsen, 2004), such as those described here.

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