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Enhanced hexose fermentation by *Saccharomyces cerevisiae* through integration of stoichiometric modeling and genetic screening

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ABSTRACT

In order to determine beneficial gene deletions for ethanol production by the yeast *Saccharomyces cerevisiae*, we performed an *in silico* gene deletion experiment based on a genome-scale metabolic model. Genes coding for two oxidative phosphorylation reactions (cytochrome c oxidase and ubiquinol cytochrome c reductase) were identified by the model-based simulation as potential deletion targets for enhancing ethanol production and maintaining acceptable overall growth rate in oxygen-limited conditions. Since the two target enzymes are composed of multiple subunits, we conducted a genetic screening study to evaluate the *in silico* results and compare the effect of deleting various portions of the respiratory enzyme complexes. Over two-thirds of the knockout mutants identified by the *in silico* study did exhibit experimental behavior in qualitative agreement with model predictions, but the exceptions illustrate the limitation of using a purely stoichiometric model-based approach. Furthermore, there was a substantial quantitative variation in phenotype among the various respiration-deficient mutants that were screened in this study, and three genes encoding respiratory enzyme subunits were identified as the best knockout targets for improving hexose fermentation in microaerobic conditions. Specifically, deletion of either *COX9* or *QCR9* resulted in higher ethanol production rates than the parental strain by 37% and 27%, respectively, with slight growth disadvantages. Also, deletion of *QCR6* led to improved ethanol production rate by 24% with no growth disadvantage. The beneficial effects of these gene deletions were consistently demonstrated in different strain backgrounds and with four common hexoses. The combination of stoichiometric modeling and genetic screening using a systematic knockout collection was useful for narrowing a large set of gene targets and identifying targets of interest.

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1. Introduction

Saccharomyces cerevisiae, or baker's yeast, is the preferred microorganism for industrial ethanol fermentation since this species has long been employed by the food industry to convert sugar into alcohol (Nevoigt, 2008). In addition to its industrial importance, bioethanol production in yeast is also an excellent model system for probing of genotype–phenotype relationships and discovering genetic perturbations for optimization of metabolic flux through central carbon metabolism (Dikicioglu et al., 2008). *S. cerevisiae* and many other organisms first catabolize hexoses (*e.g.* glucose, fructose, mannose) through the glycolytic pathway with pyruvate as the end product. In turn, pyruvate can be reduced to ethanol *via* a two-step fermentative pathway. Alternatively, in the presence of oxygen, pyruvate may be completely oxidized to carbon dioxide *via* tricarboxylic acid (TCA) cycle enzymes, which results in transfer of electrons to oxygen and ATP generation (Sonnleitner and Käppeli, 1986). It is well known that *S. cerevisiae* can grow and remain viable in anaerobic conditions without oxidative degradation of pyruvate because some ATP is generated during glycolysis. However, when even small amounts of oxygen are present, some of the carbon source will be metabolized oxidatively (cellular respiration). In Crabtree positive yeasts such as *S. cerevisiae*, a low external glucose concentration is more conducive to the respiratory metabolism than a very high glucose concentration. The cellular respiration process is more efficient than fermentation in terms of net energy payback to the cell but

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is counterproductive for the goal of optimal ethanol production. Similarly, *S. cerevisiae* can also direct carbon flux toward synthesis of other fermentation by-products such as glycerol or acetate in order to maintain redox balance within the cells (Costenoble et al., 2000; Remize et al., 2000). Therefore, we hypothesized that elimination of some unknown reactions may shift carbon flux away from the undesirable pathways (*i.e.* TCA cycle, glycerol/acetate synthesis, *etc.*) and increase the ethanol-producing capability of the organism. It is important that any deleted reaction(s) should not lead to undesirable side effects caused by unknown interactions in the system. For example, a previous metabolic engineering study described yeast knockout strains with increased ethanol yield on glucose, but the strains were accompanied by a substantial reduction in specific growth rate (Nissen et al., 2000). The objective of this study was to engineer *S. cerevisiae* toward optimal ethanol fermentation with minimal impact on growth phenotype by using a genome-scale metabolic model to systematically guide the identification of novel gene deletion targets.

The yeast *S. cerevisiae* was the first eukaryotic organism whose genome was completely sequenced and now several databases exist containing a wealth of information about genes, open reading frames, and gene products (Goffeau et al., 1996; Nevoigt, 2008). The availability of very efficient transformation methods and many genetic tools, such as specialized expression vectors and selectable markers, has facilitated advanced genetic engineering techniques (Gietz and Schiestl, 2007). In addition, an extensive collection of systems biology tools and metabolic reconstructions makes *S. cerevisiae* an amenable microorganism for *in silico* metabolic engineering. A total of six predictive genome-scale metabolic models have been created in the last 15 years to provide a mathematical framework for analyzing the metabolic potential of this organism (Nookaew et al., 2011), and the predictive power of these models has been validated against experimental data (Feist et al., 2008). Constraint-based flux analysis (or flux balance analysis) is a proven mathematical approach for application of the genome-scale models in order to predict phenotypic results and identify gene targets for metabolic engineering. This method only requires information about reaction stoichiometry, metabolic requirements for growth, and a few other strain-specific parameters without any of the difficult-to-measure kinetic parameters that are required for more theoretical approaches (Orth et al., 2010; Varma and Palsson, 1994). An optimal flux distribution representing the strain's predicted phenotype under defined conditions can be calculated based on specified reaction constraints and an objective function, which is generally accepted to be biomass maximization in microorganisms (Edwards et al., 2001; Famili et al., 2003). The main advantage of using stoichiometric modeling techniques is for providing an efficient way to narrow large sets of candidate genes without costly and time-consuming initial wet experiment screening of extensive gene sets. Inspired by the fact that stoichiometric models have been successfully applied to study and improve production of a variety of value-added products in *S. cerevisiae* (Asadollahi et al., 2009; Bro et al., 2006; Brochado et al., 2010; Ng et al., 2012), we hypothesized that a model-based approach could be used to generate new metabolic engineering strategies for enhancing ethanol fermentation in this yeast.

For accomplishing the objective, we used the genome-scale stoichiometric model *i*ND750 (Duarte et al., 2004a) to evaluate the effect of gene deletion on ethanol production in yeast. A single gene deletion simulation was conducted at various oxygen uptake rates, and the *in silico* knockout phenotypes were scanned for improvement in ethanol production while maintaining sufficient biomass for cell growth. Twenty potential gene targets linked to two enzymes in the electron transport chain (cytochrome c oxidase, ubiquinol cytochrome c reductase) were identified for further evaluation. Though stoichiometric modeling is a powerful tool for *in silico* metabolic engineering, it is important to note that neither constraint-based flux analysis nor the *i*ND750 model accounts for kinetic and regulatory effects that may significantly impact *in vivo* results. It is possible that genetic regulation may outweigh stoichiometric effects in certain situations and thus lead to unexpected results (Alper et al., 2005). To overcome this limitation and compare all knockout targets identified by the gene deletion simulation, laboratory screening was conducted in shake flask experiments with minimal media. The combination of a model-based gene targeting strategy and experimental screening led to identification of three outstanding single knockout strains for improving ethanol production on glucose in microaerobic conditions. The beneficial effects of these mutant strains were demonstrated on two other common hexoses (fructose and mannose) and a disaccharide (sucrose). Furthermore, this study describes the significant variation in phenotype among respiration-deficient mutants of *S. cerevisiae* and provides knowledge of specific genetic targets for optimizing ethanol production coupled with cell growth.

2. Materials and methods

2.1. In silico design

The *S. cerevisiae* genome-scale metabolic model *i*ND750 was used in this study (Duarte et al., 2004a). The model contains 750 genes and 1266 associated reactions, including 1149 intracellular reactions, 116 membrane exchange fluxes, and a biomass equation. Compartmentalization of 646 unique metabolites in the cell, which have been assigned to one of eight cellular compartments (cytosol, mitochondrion, peroxisome, nucleus, endoplasmic reticulum, Golgi apparatus, and vacuole), results in 1059 total species in the model that are all stoichiometrically balanced. The dimensions of the stoichiometric matrix are therefore 1059 metabolites by 1266 reactions. The exchange fluxes for the following metabolites were unconstrained in the model to assume excess for simulating minimal media conditions: NH_4^+, SO_4^-, K^+, Na^+, PO_4^{3-}, and H_2O. Glucose was the sole carbon source with an uptake rate of 5 mmol/gDW h, and the non-growth associated ATP maintenance requirement was constrained to 1 mmol/gDW h (Stouthamer, 1979). Oxygen uptake rates were constrained in the model to appropriate values for yeast fermentation in oxygen-limited conditions (see Section 3).

The COBRA (constraint-based reconstruction analysis) toolbox is a software package and valuable systems biology tool allowing for predictive computations of cellular metabolism using constraint-based models (Becker et al., 2007). All gene deletion simulations in this study were conducted using the singleGeneDeletion function in the COBRA toolbox. This function constrains the flux to zero for the reaction(s) corresponding to a deleted gene based on gene-reaction associations in the model; then, the algorithm calculates a metabolic flux distribution by applying an objective function and defined reaction constraints. The constraint-based flux analysis was performed using linear programming library GLPK (http://ftp.gnu.org/gnu/glpk/) in MATLAB (2010b, Mathworks, Natick, MA) with maximization of biomass as the objective function.

2.2. Strains and knockout strain construction

S. cerevisiae strains BY4742 (*MATalpha*, *leu2*, *his3*, *ura3*, *lys2*), CEN.PK2-1D (*MATalpha*, *leu2*, *his3*, *ura3*, *trp1*), and JAY291 (*MAT*a) (Argueso et al., 2009) were used in this study as control strains and for engineering of knockout strains. Single knockout mutants with BY4742 background were obtained from Yeast Knockout MATalpha Collection (OpenBiosystems, Lafayette, CO). The *COX9* deletion cassette with *KanMX* marker was cloned from BY4742 Δ*cox9* by PCR

Table 1
Primers used in this study.

Primer	Sequence	Comment
COX9-f	AGTTTGTGGTTGAGCAGTCG	*COX9* deletion cassette using a *KanMX* marker
COX9-r	GGCAAATTGGCAGGTATTCG	
Cd-f	CCTTCGATGGATTCGTCAGT	Confirm *COX9* deletion
KanMX-r	CTTTTCCTTACCCATGGTTGT	
QCR6-f	GCTACAATCAAGCCGCGGCTAT	*QCR6* deletion cassette using a *KanMX* marker
QCR6-r	CTACCTGCATTTCCAATGGGCG	
Q6d-f	GGTGGAAGGTATGGATATGGA	Confirm *QCR6* deletion
QCR9-f	GGGTGACGAATTTCGAGTGACT	*QCR9* deletion cassette using a *KanMX* marker
QCR9-r	GCAAAGACAACCCCAAACCCTA	
Q9d-f	TGCCATGAGAAGAGGTGATTTA	Confirm *QCR9* deletion

with primers COX9-f and COX9-r. The *QCR6* deletion cassette with *KanMX* marker was cloned from BY4742 Δ*qcr6* by PCR with primers QCR6-f and QCR6-r. The *QCR9* deletion cassette with *KanMX* marker was cloned from BY4742 Δ*qcr9* by PCR with primers QCR9-f and QCR9-r. All primers were obtained from Integrated DNA Technologies (Coralville, IA) and are listed in Table 1. The deletion cassette was then integrated into the genome of CEN.PK2-1D or JAY291 strain by transformation using the EZ-Transformation kit (BIO 101, Vista, CA) or a high efficiency lithium acetate protocol (Gietz and Schiestl, 2007). Positive transformants were selected by G418 resistance on YPD agar plates. Gene deletion was confirmed by colony PCR with confirmation primers Cd-f and KanMX-r for *COX9* deletion, primers Q6d-f and KanMX-r for *QCR6* deletion, or primers Q9d-f and KanMX-r for *QCR9* deletion (Table 1).

2.3. Media and culture conditions

To prepare yeast strains for inoculation, cells were cultivated overnight at 30 °C and 250 RPM in YPD medium (10 g/L yeast extract, 20 g/L Bacto peptone, 20 g/L glucose). Batch fermentation was carried out at 30 °C and 100 RPM in yeast synthetic complete (YSC) medium (1.7 g/L yeast nitrogen base, 5 g/L ammonium sulfate, and 0.79 g/L complete supplement mixture (MP Biomedicals, Solon, OH) for supplying amino acids and nucleobases) with 40 g/L of the appropriate sugar (glucose, fructose, sucrose, or mannose). To select yeast transformants with the *KanMX* marker, a YPD agar plate was used with 200 μg/mL of G418.

2.4. Fermentation experiments

Yeast pre-cultures were grown with 5 mL of YPD medium in an orbital shaker at 30 °C to prepare inoculums for fermentation experiments. Cells in exponential phase were harvested and inoculated after removing used YPD. Flask fermentation experiments were performed using 25 mL (or 50 mL) of YSC medium with 40 g/L of the appropriate sugar in a well-controlled shaking incubator (Thermoscientific, MaxQ4000, Dubuque, IA) under oxygen-limited conditions. Initial cell densities were adjusted to OD_{600} of ~0.1. During the course of the fermentation, the temperature and agitation rate were kept constant at 30 °C and 100 RPM, respectively.

2.5. Analytical methods

Cell concentration was measured by optical density (OD) at 600 nm using a UV-visible spectrophotometer (Biomate 3, Thermoscientific, Madison, WI). The concentration of sugar, ethanol, glycerol, and acetate in batch fermentations was determined by a high-performance liquid chromatography system (Agilent Technologies 1200 series) with a refractive index detector (RID) and a Rezex ROA-Organic Acid H^+ column (Phenomenex Inc., Torrance, CA). The column temperature was kept constant at 50 °C and the elution was performed with 0.005 N H_2SO_4 at a flow rate of 0.6 mL/min.

3. Results

3.1. In silico gene deletion simulations

The *S. cerevisiae* *i*ND750 genome-scale model accounts for 750 genes associated with 1266 metabolic reactions (including membrane exchange fluxes) and 1061 internal and external metabolites (eight cellular compartments). In order to obtain reliable predictions, it is important to consider which metabolic state(s) should be used to most accurately constrain the model. For example, the yeast *S. cerevisiae* is well known to adjust to external oxygen availability by using respiratory and/or fermentative metabolic modes (Jouhten et al., 2008); therefore, varying the oxygen uptake rate (OUR) *in silico* can have a significant effect on model predictions. Based on previous studies with a yeast genome-scale metabolic model (Duarte et al., 2004b), the oxygen uptake rate was varied from 0 to 20 mmol/gDW h to determine the effect of this variable on cellular metabolism and identify an appropriate range for model simulations. For all model simulations, glucose was the sole carbon source (uptake rate = 5 mmol/gDW h) and flux constraints were adjusted for minimal media. Under complete anaerobic conditions (OUR = 0), the *i*ND750 model predicted a low maximum specific growth rate (0.0853 h^{-1}) and high ethanol yield and productivity (0.424 g ethanol/g glucose; 8.288 mmol/gDW h), which is in accordance with fermentative metabolism. As the oxygen uptake rate increased to a saturation point (OUR = 12.5 mmol/gDW h), the model predicted a shift toward respiratory metabolism with the maximum specific growth rate increasing to its maximum value (0.478 h^{-1}) and ethanol production rate decreasing to zero. Four different oxygen uptake rates at <1 mmol/gDW h were selected to mimic microaerobic conditions for all subsequent gene deletion simulations; these values were chosen after comparing *in silico* ethanol yields with typical flask fermentation results in oxygen-limited conditions.

The gene deletion simulation was conducted at the oxygen uptake rate values of interest using the COBRA toolbox, and the *in silico* yields of ethanol, glycerol, and biomass were calculated as criteria for screening the 750 knockout mutants. In most cases, biomass yield and ethanol yield are inversely related, so knockout target selection must strike a balance between improving ethanol production and maintaining sufficient biomass synthesis for cell growth. We searched for deletion targets predicting an increase in ethanol yield while maintaining at least 80% of the wild type cell's biomass yield. Twenty such genes were identified and are listed in Table 2 with the gene name, related enzyme, and enzyme classification. All 20 genes are linked to two oxidative phosphorylation enzymes, cytochrome c oxidase (COX) and ubiquinol cytochrome c reductase (QCR), which are both critical for the cell's respiratory metabolism. The model predicts an identical flux distribution for each of the 20 knockout strains and thus an equal degree of improvement toward the objective. Fig. 1A shows a plot of the *in silico* ethanol and biomass specific productivity *versus* oxygen uptake rate for the wild-type strain and all 20 mutants

Table 2
In silico gene deletion targets for improving ethanol production while maintaining adequate biomass for cell growth.

Standard name	Systematic name	Enzyme name	Classification
COX1	Q0045	Cytochrome c oxidase (complex IV)	Oxidative Phosphorylation
COX2	Q0250		
COX3	Q0275		
COX4	YGL187C		
COX6	YHR051W		
COX7	YMR256C		
COX8	YLR395C		
COX9	YDL067C		
COX12	YLR038C		
COX13	YGL191W		
COB	Q0105	Ubiquinol 6 cytochrome c reductase (complex III)	
COR1	YBL045C		
CYT1	YOR065W		
QCR2	YPR191W		
QCR6	YFR033C		
QCR7	YDR529C		
QCR8	YJL166W		
QCR9	YGR183C		
QCR10	YHR001W-A		
RIP1	YEL024W		

from Table 1. When comparing the mutant phenotypes to the wild type, the model predictions show an increasing improvement in ethanol yield and increasing reduction in biomass yield at higher oxygen uptake rates (Fig. 1B). Under anaerobic conditions, the wild type and mutant strains have the same flux distribution. At an oxygen uptake rate of 1 mmol/gDW/h, the *in silico* ethanol yield and biomass yield of the knockout strains are 4% higher and 20% lower than the wild type, respectively. The results from the model-based simulations seem to indicate that COX-related genes and QCR-related genes may be good deletion targets for improving glucose fermentation to ethanol in oxygen-limited or fully aerobic conditions.

3.2. Experimental screening of knockout mutants

In order to validate the *in silico* results and determine the effect of various subunits on overall enzyme function, a total of 18 cytochrome c oxidase (COX) single knockout mutants and nine ubiquinol cytochrome c reductase (QCR) single knockout mutants were screened by flask fermentation in glucose minimal media. Fig. 2A and B shows the average percent change in ethanol production rate and biomass yield as compared to control strain BY4742 for each of the COX knockout strains and QCR knockout strains, respectively. The 18 COX mutants that were evaluated are deficient in one of the following genes: any of six out of the 10 COX genes identified by the *in silico* gene deletion study (Table 2) or any of 12 other COX genes not included in the *i*ND750 model. Four of the 10 COX genes identified by the *in silico* simulation were either mitochondrial genes (*COX1*, *COX2*, *COX3*) or resulted in a lethal knockout phenotype (*COX4*) and therefore could not be deleted *in vivo*. The nine QCR mutants that were evaluated are deficient in any one of the QCR genes predicted by the model-based simulation (Table 2) except for *COB*, which is a mitochondrial gene and thus could not be deleted.

Fig. 2 clearly illustrates that all deletion mutants did not have a similar phenotype. However, in qualitative agreement with the model-based predictions, 12 of the 18 COX mutants (Δ*cox6*, Δ*cox8*, Δ*cox9*, Δ*cox10*, Δ*cox12*, Δ*cox14*, Δ*cox16*, Δ*cox17*, Δ*cox18*, Δ*cox20*, Δ*cox23*, Δ*cox25*) and seven of the nine QCR mutants (Δ*cor1*, Δ*cyt1*, Δ*qcr2*, Δ*qcr6*, Δ*qcr7*, Δ*qcr9*, Δ*qcr10*) did show an improvement in ethanol production rate and a reduction in biomass yield as compared to the control strain. Among the 18 COX mutants, BY4742 Δ*cox9* stood out as the best strain for our objective with the largest increase in ethanol production rate; the ethanol-producing performance of this strain exceeded the second best COX deletion strain BY4742 Δ*cox12* by a factor of approximately two. The BY4742 Δ*cox9* strain consumed glucose at a 22% faster rate and showed a 12% increase in ethanol yield over the control strain; however, the Δ*cox9* mutant also showed a significant loss in final cell density ($P < 0.05$). The complete fermentation profiles for BY4742 and BY4742 Δ*cox9* are displayed in Fig. 3A and B, respectively. Among the nine QCR mutants, BY4742 Δ*qcr9* was the best strain in terms of ethanol production rate. The BY4742 Δ*qcr9* strain had an 18% faster glucose consumption rate and a 7% higher ethanol yield when compared to the control strain. In addition, we also observed that BY4742 Δ*qcr6* was the best of the QCR mutants in terms of ethanol production rate coupled with biomass yield. This was especially interesting because deletion of either *COX9* or *QCR9* seemed to have some negative effect on cell growth. The BY4742 Δ*qcr6* mutant showed no significant reduction in maximum specific growth rate or final cell density but still consumed glucose 17% faster and produced ethanol with a 6% higher yield than BY4742.

3.3. Evaluation of Δcox9, Δqcr6, and Δqcr9 mutations in CEN.PK2-1D background

To determine whether or not the observed improvement in ethanol yield and productivity by BY4742 Δ*cox9*, BY4742 Δ*qcr6*, and BY4742 Δ*qcr9* is strain-specific, we deleted *COX9*, *QCR6*, and *QCR9* in the laboratory strain CEN.PK2-1D. Geneticin (G418) resistance and colony PCR were used to confirm successful gene deletion. In flask fermentations with glucose minimal media, the engineered strains CEN.PK2-1D Δ*cox9*, CEN.PK2-1D Δ*qcr6*, and CEN.PK2-1D Δ*qcr9* showed improvement in ethanol production rate as compared to the control strain by 33%, 36%, and 38%, respectively. Furthermore, deletion of *COX9*, *QCR6*, and *QCR9* in CEN.PK2-1D background improved ethanol yield by 10%, 13%, and 15%, respectively. Interestingly, the CEN.PK2-1D Δ*qcr6* strain also showed no significant reduction in maximum specific growth rate or final cell density *versus* the control strain. While the Δ*cox9* and Δ*qcr9* mutants grew slower than the control during the lag phase and early exponential phase of the fermentation, the Δ*qcr6* strain maintained a similar or even slightly higher cell density. Fig. 3C and D displays the related fermentation profiles for the Δ*qcr6* and Δ*qcr9* mutants in CEN.PK2-1D background. Also, Table 3 presents a summary of the glucose consumption rate, ethanol yield, and ethanol production rate by the wild type, Δ*cox9* mutant, Δ*qcr6* mutant, and Δ*qcr9* mutant in both BY4742 and CEN.PK2-1D strains.

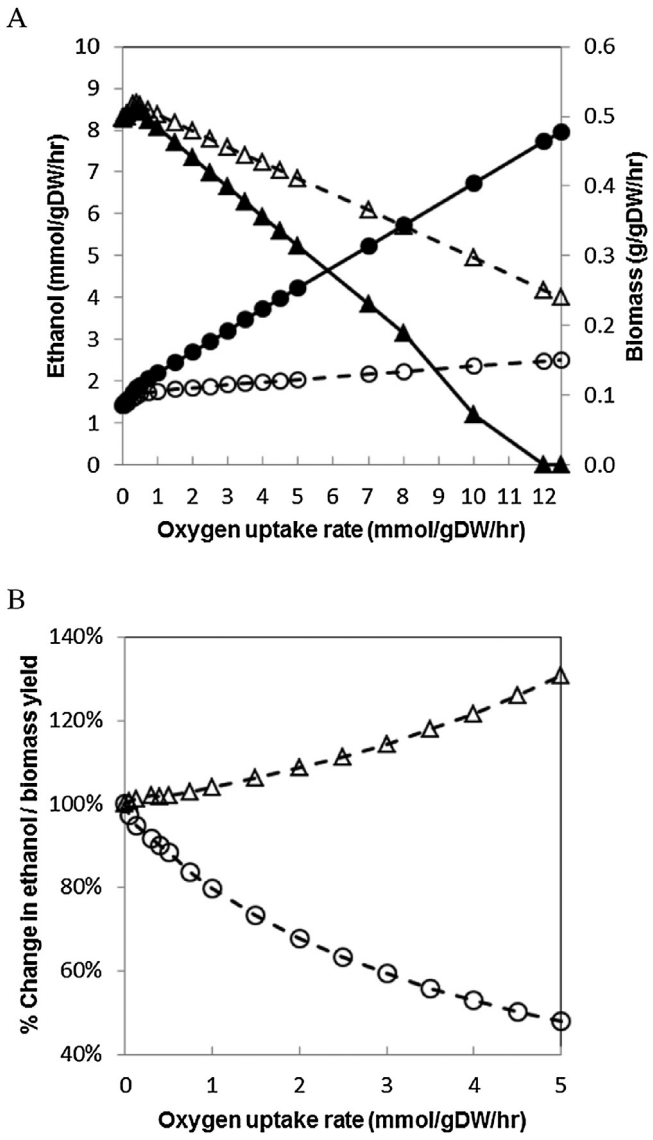

Fig. 1. (A) The *in silico* ethanol and biomass specific productivity for the wild type strain and 20 knockout mutants and (B) the percent change in ethanol and biomass yield by gene deletion as a function of oxygen uptake rate. Symbols: wild-type ethanol (closed triangle), knockout ethanol (open triangle), wild-type biomass (closed circle), and knockout biomass (open circle).

Table 3
Fermentation parameters[a] of *S. cerevisiae* wild type and Δ*cox9*, Δ*qcr6*, and Δ*qcr9* mutants in glucose with various strain backgrounds.

Strains	Glucose		
	r_{Gluc} ± SD	P_{EtOH} ± SD	Y_{EtOH} ± SD
BY4742	0.811 ± 0.009	0.275 ± 0.002	0.339 ± 0.002
BY4742 Δ*cox9*	0.991 ± 0.002	0.376 ± 0.003	0.380 ± 0.004
BY4742 Δ*qcr6*	0.952 ± 0.006	0.342 ± 0.010	0.359 ± 0.009
BY4742 Δ*qcr9*	0.959 ± 0.009	0.349 ± 0.007	0.364 ± 0.004
CEN.PK2-1D	1.312 ± 0.004	0.448 ± 0.008	0.342 ± 0.006
CEN.PK2-1D Δ*cox9*	1.586 ± 0.001	0.594 ± 0.006	0.375 ± 0.004
CEN.PK2-1D Δ*qcr6*	1.573 ± 0.002	0.608 ± 0.002	0.387 ± 0.001
CEN.PK2-1D Δ*qcr9*	1.567 ± 0.002	0.619 ± 0.003	0.395 ± 0.003

[a] Fermentation parameters were measured at 36.5 h (BY4742) or 22 h (CEN.PK2-1D) of incubation. r_{Gluc}, glucose consumption rate (g glucose/L h); P_{Etoh}, ethanol productivity (g ethanol/L h); Y_{Etoh}, ethanol yield (g ethanol/g glucose); SD means standard deviation.

The results demonstrate that the beneficial effects of these three deletions during glucose fermentation are general to *S. cerevisiae* and not specific to any particular strain.

3.4. Evaluation of Δ*cox9*, Δ*qcr6*, and Δ*qcr9* mutants on other hexoses

Fructose, sucrose, and mannose are three other hexoses abundant in nature that can be fermented to ethanol by *S. cerevisiae*. We were interested to evaluate the identified single knockout mutants on these three hexoses to determine if the improvement in ethanol production rate and ethanol yield on glucose would extend to other sugars with a similar metabolism as glucose. Therefore, we conducted another series of flask fermentations with the knockout strains BY4742 Δ*cox9*, BY4742 Δ*qcr6*, and BY4742 Δ*qcr9* in minimal media with 40 g/L of fructose, sucrose, or mannose as the sole carbon source. The ethanol production rate, ethanol yield, and final cell density for the wild type and all three mutant strains is summarized in Fig. 4A, B, and C, respectively; the figure includes results for all four hexoses used in this study including glucose results discussed previously. Fig. 4A shows that each of the three knockout strains showed a significant improvement ($P < 0.05$) in ethanol production rate on glucose, fructose, mannose, or sucrose. Furthermore, all of the knockout strains had a higher average ethanol yield than the control on each carbon source (Fig. 4B); the BY4742 Δ*qcr6* strain on glucose was the only case where this improvement in ethanol yield was not statistically significant ($P = 0.087$). Finally, the BY4742 Δ*cox9* strain had a significant reduction ($P < 0.05$) in final cell density for each of the four hexoses while BY4742 Δ*qcr6* had no significant change in final cell density for any hexose (Fig. 4C). The results clearly demonstrate that the outstanding characteristics of the three knockout strains identified in this study may be extended to a variety of fermentable sugars in microaerobic conditions.

3.5. Growth on non-fermentable carbon source

The Δ*cox9*, Δ*qcr6*, and Δ*qcr9* mutants were streaked onto YP glycerol plates to compare growth on a non-fermentable carbon source. Fig. 5 shows the results in both BY4742 and CEN.PK2-1D background. In both cases, the Δ*cox9* and Δ*qcr9* mutants were not able to grow at all, which indicates a complete respiration deficiency by these knockout strains. However, the Δ*qcr6* mutant did show some growth on glycerol, although the colony size was considerably smaller than for the wild-type strain. This indicates only a partial respiration deficiency by deleting *QCR6* and explains why the Δ*qcr6* mutant was able to grow better than either Δ*cox9* or Δ*qcr9* during hexose fermentation.

3.6. Industrial strain fermentation with high initial glucose concentration

In order to evaluate the knockout strains in a condition where more fermentation takes place, we conducted another experiment using the industrial *S. cerevisiae* strain JAY291, which consumes glucose much faster (2.93 g/L h) and also produces ethanol much faster (1.21 g/L h) than either of the laboratory strains used previously (BY4742 or CEN.PK2-1D). We proceeded to delete *QCR6* and *QCR9* in the JAY291 background and then evaluated the mutants (Δ*qcr6* and Δ*qcr9*) in minimal media with a high initial glucose concentration (100 g/L). We chose to omit *COX9* deletion in JAY291 since deletion of *QCR9* and deletion of *COX9* resulted in a similar growth phenotype for the laboratory strains. Fig. 6 shows the results for the high-sugar industrial strain fermentation. Although the improvement by our knockout mutants is reduced in these highly fermentative conditions, we still see a higher average ethanol production rate (9–12%) and higher glucose consumption rate (9–10%) by JAY291 Δ*qcr6* or JAY291 Δ*qcr9* as compared to the control. For

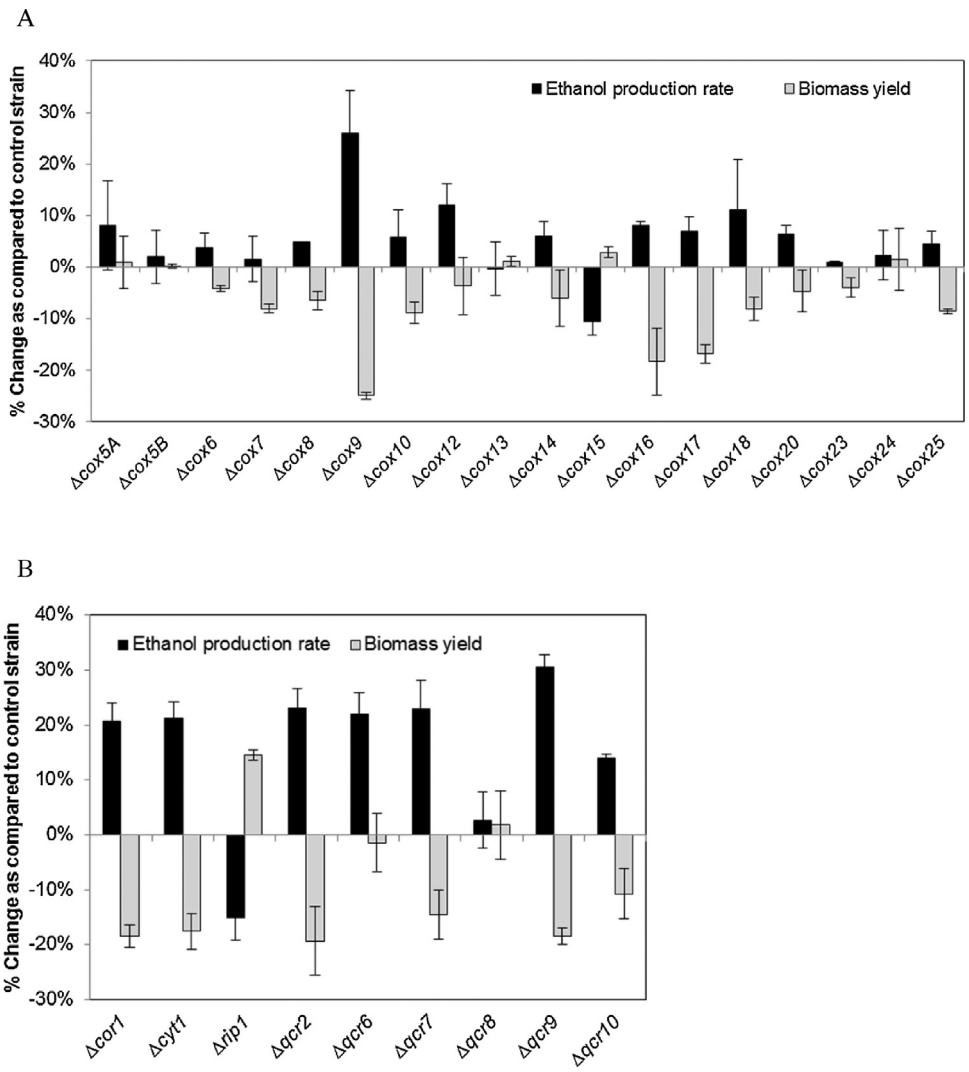

Fig. 2. A total of (A) 18 cytochrome c oxidase single knockout mutants and (B) nine cytochrome c reductase single knockout mutants were screened by glucose fermentation in minimal media. The average percent change in ethanol production rate and biomass yield is displayed for each knockout mutant as compared to control strain BY4742. All results are the average of duplicate experiments and the error bars represent one standard deviation.

both mutants, the improvement in glucose consumption rate was statistically significant ($P < 0.05$). However, the increase in average ethanol production rate was not statistically significant at the 95% confidence interval for JAY291 Δ*qcr6* ($P = 0.109$) or JAY291 Δ*qcr9* ($P = 0.055$). There was almost no improvement in ethanol yield by the knockout strains under these conditions. The JAY291 Δ*qcr6* and JAY291 Δ*qcr9* strains both grew as well as the control throughout the fermentation and actually showed a slightly higher final cell density.

4. Discussion

Rapid and efficient hexose fermentation by *S. cerevisiae* with maximum yield and productivity of ethanol is important for many food and fuel applications. In addition, the ethanol production pathway is also an excellent model system for optimization of metabolic flux through central carbon metabolism. In this study, we employed an *in silico* genome-scale metabolic model to identify and select knockout targets for improving ethanol production in yeast while maintaining acceptable overall growth rate. The model-based strategy allowed us to narrow an extensive gene set and systematically guided us to consider genes encoding two oxidative phosphorylation enzymes, which catalyze the last two reactions in the respiratory electron transport chain. Ubiquinol cytochrome c reductase (EC 1.10.2.2) and cytochrome c oxidase (EC 1.9.3.1), also known as complex III and complex IV in the electron transport chain, are integral membrane proteins in the mitochondrial membrane which are critical for aerobic life due to their ability to maintain a proton gradient and thus synthesize ATP (Brunori et al., 2005). In *S. cerevisiae*, it is known that the ubiquinol cytochrome c reductase (QCR) enzyme contains ten non-identical subunits and is responsible for transferring electrons from reduced ubiquinone to ferricytochrome c (Shi et al., 2001). Subsequently, the cytochrome c oxidase (COX) enzyme, which possesses eleven distinct subunits and depends on a number of other related proteins for assembly and activity, transfers electrons from ferrocytochrome c to oxygen and thus catalyzes the reduction of oxygen to water (Geier et al., 1995). The *Saccharomyces* Genome Database (Cherry et al., 2012) contains a wealth of information about genes that are directly or indirectly associated with these two multi-subunit enzymes. In this study, the stoichiometric model-based simulation predicted that deletion of any one of the multiple genes encoding the COX enzyme or QCR enzyme would have an identical effect on phenotype (Fig. 1). While stoichiometric modeling was useful for narrowing a large set of gene targets, this approach was not sufficient to distinguish between multiple genes linked to a single enzyme because the

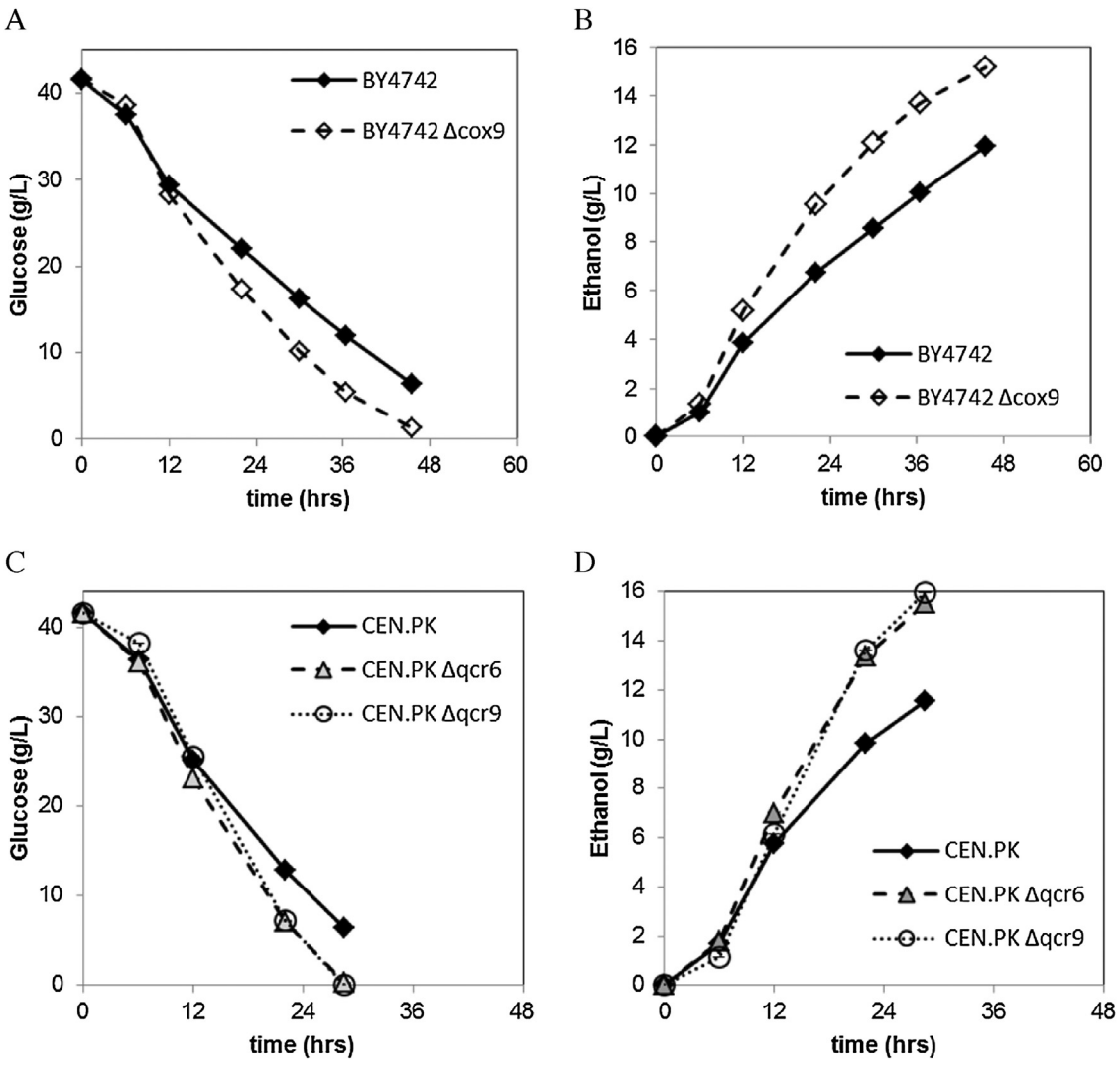

Fig. 3. Fermentation profiles for glucose consumption and ethanol production during flask fermentation by *S. cerevisiae* strains: (A and B) BY4742 and BY4742 Δ*cox9*, (C and D) CEN.PK2-1D, CEN.PK2-1D Δ*qcr6*, and CEN.PK2-1D Δ*qcr9*. All results are the average of duplicate experiments. The error bars represent one standard deviation and are not visible when smaller than the symbol size.

metabolic model does not account for genetic regulation and other unknown potential cellular interactions. Distinguishing between the 20 gene targets in Table 2 is where the utility of the *in silico* model ends and the need for experimental screening begins. To the best of our knowledge, no previous work has been done to evaluate and compare the phenotypic effect in *S. cerevisiae* by *in vivo* deletion of each one of the QCR-related genes or COX-related genes identified by our model-based gene targeting strategy.

Eighteen COX single knockout mutants and nine QCR single knockout mutants were screened by glucose fermentation in oxygen-limited conditions to evaluate the model predictions. Over two-thirds of the knockout mutants identified by the *in silico* study exhibited experimental behavior qualitatively consistent with model predictions; however, the notable exceptions illustrate the danger of using a purely stoichiometric model-based approach. The experimental screening results demonstrate a high degree of variability among the phenotypes of QCR and COX single knockout mutants (Fig. 2) and indicate that the individual subunits contribute to the overall function of the enzyme complexes in very different ways. For the nine QCR single deletion mutants, the average change in ethanol production rate was a 16% increase and the average change in biomass yield was a 9% decrease as compared to the control strain. This result indicates a qualitative fit of experimental data with model predictions. However, the best QCR-related ethanol-producing strain (Δ*qcr9*) increased ethanol production rate by 27% and reduced biomass yield by 20%; the worst QCR-related ethanol-producing strain (Δ*rip1*) decreased ethanol production rate by 15% and increased biomass yield by 15%. The model-based gene targeting strategy was unable to predict these variations among QCR-related gene deletion mutants or to identify the most outstanding single knockout mutants. The data in Fig. 2, showing the variable effect of QCR and COX gene deletions on ethanol production rate and biomass yield, could be used to build a more accurate genome-scale metabolic model of *S. cerevisiae*.

Many previous studies have reported that utilizing respiration-deficient yeast strains, or petite mutants, can improve ethanol production efficiency (Bacila and Horii, 1979; Hutter and Oliver, 1998; Kim et al., 2010). Petite mutants include any yeast strain with a nuclear DNA mutation resulting in inability to utilize non-fermentable carbon sources such as ethanol, glycerol, or acetate. Petite mutants can still grow on glucose or other fermentable carbon sources but form smaller colonies than wild type cells (Tzagoloff and Dieckmann, 1990). For industrial ethanol production, two of the major advantages of respiration deficiency include: (i) elimination of oxidative metabolism of sugars and other fermentation products and (ii) more leniency in fermentation conditions

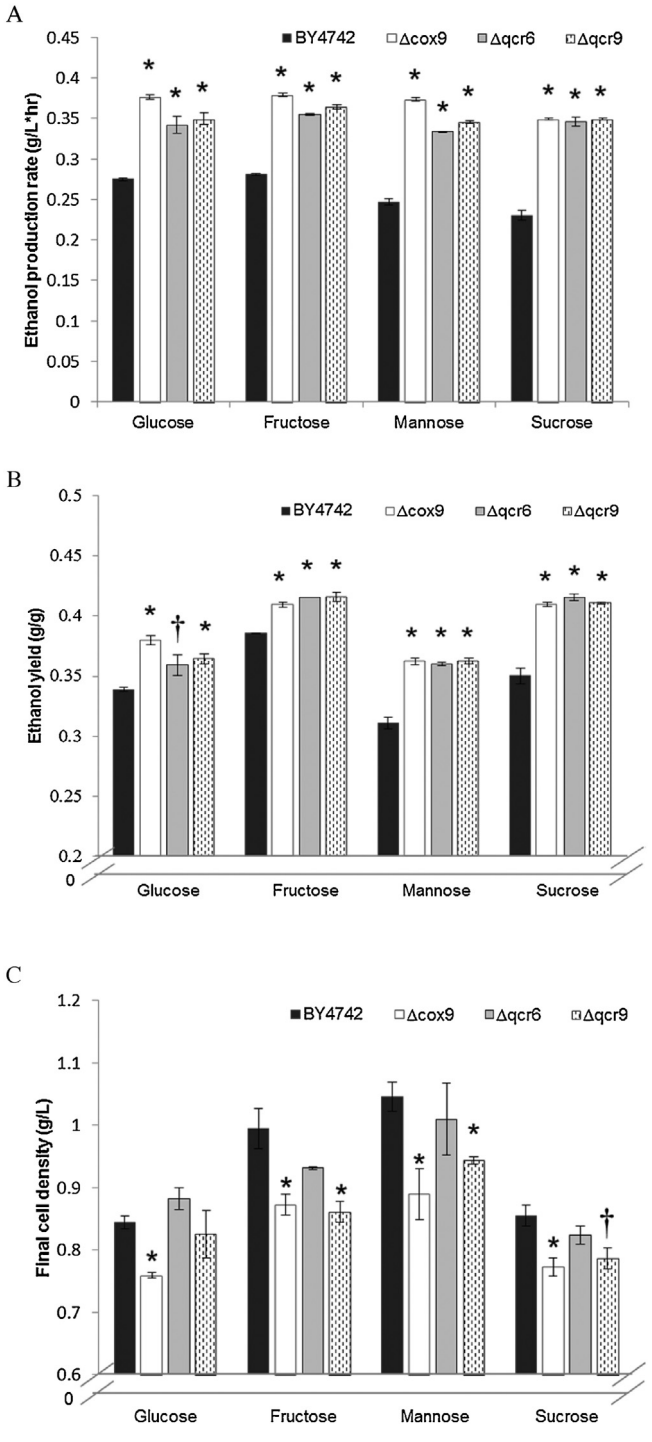

Fig. 4. Comparison of (A) ethanol production rate, (B) ethanol yield, and (C) final cell density by laboratory strain BY4742 and knockout strains BY4742 *Δcox9*, BY4742 *Δqcr6*, and BY4742 *Δqcr9* during fermentation in minimal media with four different carbon sources: glucose, mannose, fructose, and sucrose. All results are the average of duplicate experiments and the error bars represent one standard deviation. The student's *t* test was used to establish significant differences between fermentations conducted with the knockout strains and the control strain. $^{*}P < 0.05$ (95% confidence). $^{\dagger}P < 0.10$ (90% confidence).

since a strict anaerobic environment is no longer required. Though it is well known in a general sense that respiration inhibits fermentation performance, knowledge is lacking of phenotype variation among respiration-deficient mutants and specific genetic targets for maximizing ethanol production with minimal impact on growth phenotype. There are numerous approaches (genetic or chemical) to generate respiration-deficient mutants of *S. cerevisiae*, and all of the resulting strains are not equivalent. For example, some respiration-deficient mutants may have better fermentation performance than others, as illustrated by the large variation in ethanol production among 20 knockout strains identified and screened in this study (Fig. 2). Also, many respiration-deficient mutants are accompanied by a significant penalty in terms of cell growth, but the growth defect may be quite different among these strains (Fig. 2). In this study, the model-based gene deletion simulation indicated that COX-related or QCR-related deletion targets are better than other components of the respiratory system (*e.g.* NADH dehydrogenase, succinate dehydrogenase, fumarase, *etc.*) for improving ethanol production and maintaining sufficient biomass production. By experimental screening of the COX- and QCR-related mutants, we identified three specific knockout strains with maximum ethanol production and little or no growth penalty (Δ*cox9*, Δ*qcr6*, and Δ*qcr9*). Previously, it was reported that *COX9* exists as a single copy in haploid strains of *S. cerevisiae*, produces one major transcript, and encodes subunit VIIa of the COX enzyme complex, which is essential for respiratory function (Wright et al., 1986). Similarly, QCR9p, the smallest subunit of the QCR complex, is critical for correct structure of the enzyme and thus necessary for respiratory growth; its deletion led to elimination of electron transfer activity at the ubiquinol oxidase site (Graham et al., 1992; Phillips et al., 1990). In another study, QCR6p was demonstrated necessary for activity of the QCR complex only at high temperature (37 °C), but the deletion mutant showed normal respiratory growth at 30 °C (Yang and Trumpower, 1994). The present study is the first to describe the Δ*cox9*, Δ*qcr6*, and Δ*qcr9* strains in terms of ethanol-producing capability and to demonstrate the superlative characteristics of these three knockout mutants among many others for improving hexose fermentation in *S. cerevisiae* under microaerobic conditions. The Δ*qcr6* mutant was particularly interesting with a 24% improvement in ethanol production rate and no growth defect. Future work will be needed for elucidating the molecular mechanism to explain why eliminating specific protein subunits (Qcr6p, Qcr9p, or Cox9p) from the QCR or COX enzyme complex has a more beneficial influence on ethanol production and biomass yield than removing other subunits or related proteins.

We have demonstrated that the benefits of deleting *COX9*, *QCR6*, or *QCR9* in *S. cerevisiae* for ethanol production in limited-oxygen conditions are consistently observed in different strain backgrounds and extend to common hexoses such as glucose, fructose, mannose, or sucrose (Fig. 4). Even the industrial strain fermentation with high initial glucose concentration showed an increase in average ethanol production rate and glucose consumption rate by the knockout strains tested; however, it is also important to note that improvements were significantly reduced in these highly fermentative conditions (Fig. 6). First of all, we speculate that a major reason for this is because the industrial strain has already been evolved over a long time period to ferment sugar in high concentrations more efficiently. Accumulated mutations in the industrial strain increase fermentation rate and efficiency while minimizing the effects seen by deleting respiration-related genes in this background. Furthermore, extreme sugar concentration or strict anaerobic conditions will lessen the beneficial effects by the knockout strains described in this study. It is well known that excess glucose represses respiration and contributes to highly fermentative metabolism in *S. cerevisiae* even in the presence of oxygen (*i.e.* Crabtree effect). Also, strict anaerobic conditions will certainly minimize respiration and maximize fermentation by yeast. In these situations, *S. cerevisiae* performs fermentation very well, and there is often little or no room for improvement by metabolic engineering. Still, some advantage could come from engineering strains that assure optimal fermentation in various conditions and does so regardless of changing process parameters such as dissolved

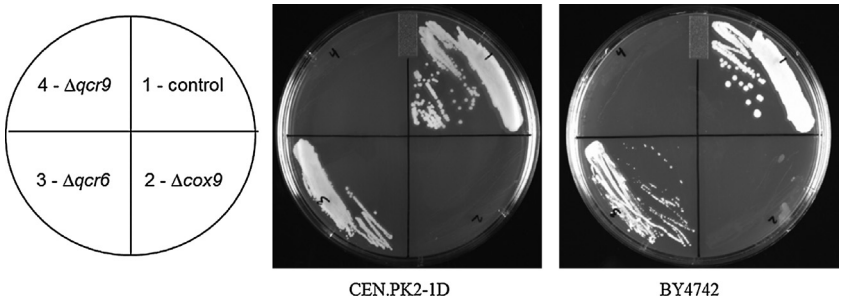

Fig. 5. Wild-type *S. cerevisiae* with CEN.PK2-1D background or BY4742 background and single knockout mutants of cytochrome c oxidase (Δ*cox9*) and cytochrome c reductase (Δ*qcr6*, Δ*qcr9*) were grown on YP glycerol agar plates. Plates were incubated at 30 °C for 3 days (CEN.PK2-1D) or 6 days (BY4742).

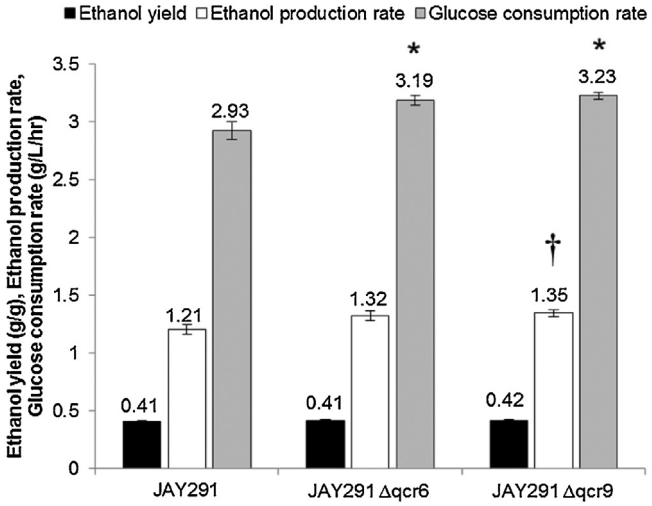

Fig. 6. Comparison of ethanol yield, ethanol production rate, and glucose consumption rate by industrial strain JAY291 and knockout strains JAY291 Δ*qcr6* and JAY291 Δ*qcr9* during fermentation in minimal media with high initial glucose concentration. All results are the average of duplicate experiments and the error bars represent one standard deviation. The student's *t* test was used to establish significant differences between fermentations conducted with the knockout strains and the control strain. $^{*}P < 0.05$ (95% confidence). $^{\dagger}P < 0.10$ (90% confidence).

oxygen or sugar concentration. For example, some applications may benefit from improved fermentation at lower sugar concentrations under continuous culture conditions. Also, many large-scale fermentations do not begin with a strict anaerobic environment (*i.e.* no reactor purging, no nitrogen charging, no tight sealing of the vessel). Thus, the fermentation will behave anaerobically only after all initial dissolved oxygen is consumed by the cells, which means that substantial process time in a microaerobic state is possible. In changing or non-ideal fermentation conditions, the Δ*qcr6* mutant may offer particular advantages due to faster ethanol production rate, higher yield, and no biomass penalty.

In conclusion, we emphasize that the main significance of the present study lies in the methodology used. The single knockout strains described in this work are quite beneficial in certain conditions (microaerobic, lower external sugar concentration), but we cannot claim to have the best ever ethanol-producing *S. cerevisiae* strains. Still, the combination of genome-scale metabolic modeling with genetic screening using a systematic knockout collection could prove to be valuable in many other cases for reducing a large set of gene targets and identifying specific targets of interest.

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