Doctoral Thesis

Evolutionary and metabolic engineering of xylose metabolism in Saccharomyces cerevisiae

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Evolutionary and metabolic engineering of xylose metabolism in *Saccharomyces cerevisiae*

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH
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Doctor of Natural Science

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table of contents</td>
<td>5</td>
</tr>
<tr>
<td>Summary/Sommario</td>
<td>7</td>
</tr>
<tr>
<td>Chapter 1 – General introduction</td>
<td>11</td>
</tr>
<tr>
<td>Chapter 2 – Evolutionary engineering of <em>Saccharomyces cerevisiae</em> for anaerobic growth on xylose</td>
<td>33</td>
</tr>
<tr>
<td>Chapter 3 – The molecular basis for anaerobic growth of <em>Saccharomyces cerevisiae</em> on xylose investigated by global gene expression and metabolic flux analysis</td>
<td>57</td>
</tr>
<tr>
<td>Chapter 4 – Investigating three potential bottlenecks of yeast xylose catabolism by rational and evolutionary engineering</td>
<td>83</td>
</tr>
<tr>
<td>Chapter 5 – Metabolic engineering of a phosphoketolase pathway for pentose catabolism in <em>Saccharomyces cerevisiae</em></td>
<td>101</td>
</tr>
<tr>
<td>Chapter 6 – Fermentation performance of engineered and evolved xylose-fermenting <em>Saccharomyces cerevisiae</em> strains</td>
<td>121</td>
</tr>
<tr>
<td>Chapter 7 – Evolutionary engineering of <em>Escherichia coli</em> for increased metabolic activity in stationary phase</td>
<td>141</td>
</tr>
<tr>
<td>Conclusions</td>
<td>159</td>
</tr>
<tr>
<td>Curriculum vitae</td>
<td>163</td>
</tr>
</tbody>
</table>
SUMMARY

The success of classical metabolic engineering strategies to improve cell factories for biotechnological purposes is strongly dependent on the availability of precise information about the system-wide interactions that determine the phenotype. Despite the massive data sets generated by genome-wide analytical techniques, system-wide understanding is often not achieved, at least not to an extent that phenotypes can be improved directly. This thesis attempts to improve primarily one such phenotype: xylose metabolism in *Saccharomyces cerevisiae*, which is important to establish an efficient fermentation process producing bio-ethanol from hemicellulose rich and cheap lignocellulosic material.

Despite intense research over the last decade, metabolic engineering strategies achieved only partial success in establishing xylose metabolism in *S. cerevisiae* and failed completely to enable anaerobic growth on this substrate. In this thesis, I present evolutionary engineering of the first yeast that is capable of strict anaerobic growth on sole xylose (Chapter 2). This unique *S. cerevisiae* mutant was then analyzed by genome-wide transcript level and metabolic flux analysis to reveal the mechanisms that hamper anaerobic growth on xylose of rationally engineered strains (Chapter 3). In particular, I provide evidence that the ultimate problem resides in the insufficient rate of glycolytic ATP formation, which in turn is reduced when NADH re-oxidation is problematic and leads to the accumulation of the reduced intermediate xylitol. The results indicate that further bottlenecks that may become relevant as soon as sufficient NADH re-oxidation is provided, might reside in xylose transport, the xylose catabolic pathway itself, and the pentose phosphate pathway (Chapter 3 and 4).

To provide a new possibility for NADH re-oxidation, I expressed a functional soluble transhydrogenase (Chapter 4) in xylose fermenting yeast. This enzyme, however, operated toward NADPH instead of the desired NADH re-oxidation, increasing even more NADH production. In a second attempt, I was able to attain NADH re-oxidation with the establishment of a functional phosphoketolase pathway (Chapter 5). With this strategy, I significantly increased the ethanol yield at the expenses of xylitol formation, and could increase simultaneously the xylose fermentation rate by additionally deleting the constitutive cytosolic aldehyde dehydrogenase gene *ALD6*, which is principally responsible for acetate accumulation.

Chapter 6 then affords a quantitative comparison of eight recently engineered or evolved yeast strains with respect to their xylose fermentation performance and robustness. Generally, industrial strains fermented xylose faster and were more tolerant to lignocellulose
hydrolysates, but accumulated more xylitol than laboratory strains. The best xylose fermenting strains were those obtained by evolutionary engineering.

Finally, I addressed a general process problem that is also relevant for ethanol production: high metabolic activity in the absence of growth (Chapter 7). In particular, I describe a new enrichment procedure toward this phenotype in an extremely slow-growing chemostat culture. I isolated thereby an *Escherichia coli* mutant with doubled catabolic rate that was maintained for at least 40 h in nitrogen starvation induced stationary phase.
SOMMARIO

La disponibilità di informazioni dettagliate, riguardanti il funzionamento dei meccanismi molecolari che determinano il fenotipo di un organismo, è essenziale per il successo di strategie classiche d’ingegneria del metabolismo miranti al miglioramento delle caratteristiche cellulari per scopi biotecnologici. Nonostante l’enorme quantità di dati generata tramite l’impiego di tecniche d’analisi a livello genomico, una comprensione sistemica completa in grado di permettere direttamente il miglioramento di vari fenotipi, non è stata ancora raggiunta. In questa tesi cerco di migliorare principalmente uno di questi fenotipi: Il metabolismo dello xilosio nel lievito *Saccharomyces cerevisiae*. Quest’ultimo è particolarmente importante per lo sviluppo di processi di fermentazione atti alla produzione di bioetanolo da scarti di natura lignocellulosica.

Nonostante l’intenso sforzo di ricerca nell’ultimo decennio, strategie tradizionali di ingegneria del metabolismo sono riuscite a raggiungere un successo solo parziale nell’instaurare una via catabolica dello xilosio in *S. cerevisiae*. Inoltre, hanno completamente fallito nel generare ceppi capaci di crescere su xilosio in condizioni strettamente anaerobiche. In questa tesi, presento la generazione tramite ingegneria evolutiva del primo ceppo di lievito capace di crescere in queste condizioni (Capitolo 2). Per rivelare i fattori che impediscono tale fenotipo in ceppi modificati con strategie tradizionali, ho quantificato l’espressione di geni a livello genomico e i flussi metabolici intracellulari del carbonio in questa mutante unica in natura (Capitolo 3). In particolare, presento osservazioni indicanti che il problema fondamentale è costituito dall’insufficiente velocità di produzione glicolitica di ATP. Quest’ultima è a sua volta ridotta quando, in condizioni anaerobiche, la riossidazione del NADH diventa problematica e causa quindi l’accumulazione del prodotto intermedio xilitolo. I miei risultati indicano che altri fattori potrebbero diventare limitanti allorquando un sufficiente grado di riossidazione del NADH potesse essere garantito. Tra questi figurano il trasporto dello xilosio, la stessa via catabolica dello xilosio e la via del pentosio fosfato (Capitoli 3 e 4).

Per fornire una nuova possibilità di riossidazione del NADH, ho espresso una transidrogenasi solubile in un ceppo di lievito modificato, capace di metabolizzare lo xilosio (Capitolo 4). Invece di riossidare l’NADH producendo NADPH, però, quest’enzima ha catalizzato la reazione inversa, aumentando ulteriormente la produzione di NADH. In un secondo tentativo, sono riuscito a raggiungere un significante grado di riossidazione del NADH implementando la via della fosfochetolasi nel lievito (Capitolo 5). Con questa
strategia, sono riuscito ad aumentare la resa dell’etanolo a scapito della produzione di xilitolo. Inoltre, sono riuscito ad incrementare simultaneamente la velocità di fermentazione dello xilosio introducendo una delezione del gene dell’ aldeide deidrogenasi \textit{ALD6}.

Il Capitolo 6 offre un’analisi comparativa delle caratteristiche di fermentazione dello xilosio e della resistenza di otto ceppi modificati o evoluti recentemente. In generale, i ceppi industriali fermentano lo xilosio più rapidamente e sono più resistenti all’effetto inibitorio della materia lignocellulosica idrolizzata, ma accumulano una maggiore quantità di xilitolo rispetto ai ceppi di laboratorio. I ceppi con le caratteristiche di fermentazione migliori sono quelli ottenuti tramite ingegneria evolutiva.

Infine, ho affrontato una questione di carattere più generale, rilevante anche per la produzione di etanolo: il mantenimento di un’ attività metabolica elevata in assenza di crescita (Capitolo 7). In particolare, descrivo un nuovo metodo di selezione di tale fenotipo in una coltura continua con una velocità di crescita estremamente bassa. Tramite questo metodo sono riuscito ad isolare una mutante di \textit{Escherichia coli} con una velocità catabolica raddoppiata per almeno 40 ore nella fase stazionaria indotta dall’assenza di azoto.
General introduction
EVOLUTIONARY ENGINEERING

The improvement of biotechnologically relevant cellular functions by recombinant DNA technology are referred today as **metabolic engineering** (9). Since the success of such rational strategies is highly dependent on precise knowledge of the molecular and functional mechanisms of a desired cellular trait, the improvement of more complex and not fully understood phenotypes is often difficult.

A rather similar situation was encountered by rational protein engineering (78), where directed evolution was successfully employed to improve existing protein functions and also to tailor new enzyme properties in the absence of functional information (7, 8). The basic concept of directed evolution, repeated cycles of random mutagenesis and screening for a single best performer, has been widely used also in classical and empirical strain development for industrial applications (65, 77). Due to the enormous amount of variants that have to be analyzed for the desired improvement, however, this approach works only if the improved phenotype can be detected by a simple screening method. The exploitation of the natural principles of evolution, mutagenesis and survival of the fittest, allows to circumvent this problem by substituting or preceding manual or automated screening with an enrichment step toward the desired phenotype. The enrichment of defined variants by the action of environmental factors for extended time periods is termed **continuous selection**, and circumvents repeated passages through the single variant level since selection is performed with populations consisting easily of more than $10^{10}$ cells. Differently from proteins, this is possible in microorganisms because they are self-replicating so that the phenotype is coupled to the genotype. Moreover, if a suitable continuous selection pressure can be applied to a large population, the continuous interplay of continuous selection and natural genetic variability is potentially able to generate more complex phenotypes that need a higher number of mutations to generate a relevant phenotype improvement (e.g. gain of function mutants). The rational application of suitable mutagenesis and continuous selection procedures toward the improvement of a specific, biotechnologically relevant microbial trait is referred as **evolutionary engineering** (13, 80). The concept of evolutionary engineering was already successfully applied for the improvement of various desirable traits like improved resistance to environmental stressing factors (13, 83), improved production properties (84, 89), as well as for novel catabolic activities (5, 81).
Mutagenesis

For evolutionary engineering it is often desirable to generate a suitable degree of genetic variability before and/or during the continuous selection process. For that purpose, chemical or radiation induced mutagenesis offer an easy and well established way (26). These methods, however, need to be calibrated for each organism to find a compromise between an appropriate level of genetic mutagenesis and cell killing. Moreover, they can not be applied continuously during the selection procedure. However, spontaneous mutagenesis occurs naturally also during continuous selection, and can be increased by appropriate environmental and physiological changes (58) or by the use of mutator strains that exhibit extraordinary high rates of mutation (14, 83). A further possibility is provided by tagged mutagenesis that is based on the introduction of randomly integrating transposable elements within the genome. This event causes gene disruption or overexpression, depending on the transposon type (18, 88). The advantage of tagged mutagenesis is the possibility to easily track the genetic changes conferring the desires phenotype, since the transposon can rapidly be localized in the genome. Genome shuffling, which allows the recombination of genomic DNA fragments between multiple and improved parents at each generation, can be finally used to generate combinatorial libraries of new strains (91, 115). The advantage of genome shuffling is the accelerated combination of positive and the elimination of negative mutations in one single strain. This method, however, needs the availability of a set of already improved mutants, which are normally generated by random mutagenesis, selection and/or screening. Hence, the application of this technique optimally follows evolutionary engineering of a starting strain that is used to generate a pool of differently improved variants.

Continuous selection

Continuous selection for evolutionary engineering purposes can be achieved with a wide variety of cultivation techniques, ranging from solid medium (60, 83), batch (55, 103) to chemostat cultures (22, 59, 90). While batch-based selection procedures favor the evolution of strains with shorter lag phases and higher maximum growth rates (53), long-term chemostat cultures increased substrate affinity and efficient conversion of the growth limiting substrate to biomass (22). Chemostats are very useful in evolutionary engineering, since they allow to maintain a desired physiological status (e.g. growth rate or limiting nutrient) for extremely long periods of time without intense supervision. Additionally, for long-term evolution experiments, simple design chemostat culture systems may be employed, which allow low
cost operation (21). Chemostats, however, have the disadvantages to potentially select for co-metabolism (36, 75). This phenomenon can be observed when the evolving population is constituted by a primary phenotype with improved limiting-substrate scavenging properties and by one or more secondary populations which are specialized in the catabolism of the by-products secreted by the primary population. Furthermore, every mutation that augments the residence time in the reactor is selected in chemostat cultures, although it doesn’t cause improved physiology (e.g. adhesion to the bioreactor walls).

**Evolution**

The process of evolution of a strain toward the achievement of an improved phenotype can be visualized in a fitness landscape, which is the quantitative representation of the strain fitness as a function of all possible genotypes that the strain could potentially assume (110, 111). In particular, all possible genotypes are represented by a space containing all possible DNA or protein sequence variants for a given organism. In this view, evolution can be conceived as adaptive walks towards the fitness landscape that are driven by incremental increases in strain fitness under the considered conditions. During natural selection, these adaptive walks end when the nearest fitness peak is reached. Since many different sequence combinations or genotypes will result in strain fitness increases, there will be many fitness peaks, but only one may represent the overall optimum. Since the principle of natural evolution does not permit adaptive walks across valleys of maladapted intermediate states from one peak to a higher one, the achievement of global optimum peaks can only be accomplished by jumps from one peak to the other. Such a possibility is provided by recombination, which combines already improved mutant forms to achieve an overall better phenotype.

A similar issue is represented by the distance between the starting point of evolution and the nearest fitness peak in the sequence space. This can be interpreted as the necessity for multiple mutations to achieve a selectable improvement toward the desired phenotype. A practical example of this problem can be seen in the introduction of a novel metabolic pathway in an organism lacking the corresponding enzymes. The modification of already available sequences to achieve the desired genotype would require many generations, and may not be possible in many cases, since too many multiple mutations have to occur in order to achieve a phenotype improvement that can be favored by the selection pressure. Nature approaches this problem by horizontal DNA transfer and recombination, thus providing heterologous sequences that may be more similar to the needed ones. In evolutionary
engineering this jump can be attained by metabolic engineering, when corresponding sequences are introduced in the desired organism. Evolutionary engineering would then serve for the optimization of pathway operation. Thus, evolutionary engineering is a complementing rather than a competing technique of metabolic engineering, and increasingly acquires its place in the metabolic engineering toolbox (Figure 1).

**Starting strains**

![Diagram showing the integration of evolutionary engineering with metabolic engineering and screening for the generation of a strain with suitable characteristics for an industrial process. Gray arrows indicate inverse metabolic engineering strategies.]

Figure 1. Schematic view of the integration of evolutionary engineering with metabolic engineering and screening for the generation of a strain with suitable characteristics for an industrial process. Gray arrows indicate inverse metabolic engineering strategies.
Screening and single mutant analysis

The isolation of single mutants with highly improved phenotypes from evolved populations has become also possible for more complex phenotypes, which can hardly be assessed on plate or by simple assays, but need more complicated physiological analysis. The recent development of miniaturized cultivation systems allows the parallel physiological analysis of hundreds or thousands of single variants (20), which may be enough to assess the clonal composition of an evolved population where the desired phenotype is assumed to be well represented. Once identified and isolated by screening, single mutants can be used directly for industrial application and/or serve as model organisms to elucidate the molecular basis of a desirable trait.

Starting from clones with the desired phenotype, novel genome-wide techniques like mRNA level (transcriptome) (17), protein level (proteome) (42) and low molecular weight metabolite level (metabolome) (25) analysis provide the tools for molecular and functional analysis of the causes for the desired phenotype. Among these whole cell analysis techniques, we can find also metabolic flux analysis, which is a method to assess the rates of intracellular reactions that is very useful if microbial traits with metabolic relevance are addressed (92, 97). Starting from data on uptake and secretion rates, biosynthetic requirements, quasi-steady state mass balances on intracellular metabolites, and assumptions about metabolic stoichiometry, this technique allows to compute the intracellular carbon flux distribution. More recently, the use of $^{13}$C-labeled substrates increased the accuracy of this technique by avoiding or validating critical assumptions (93, 108). Thus, metabolic flux analysis provides insights, which in combination with the other genome wide techniques, may accelerate the identification of previously unrecognized critical factors and bottlenecks which hamper engineering of useful metabolic industrial traits.

Engineering of novel or improved strain properties by applying the knowledge acquired through the analysis of the desired phenotype in other strains found in nature or obtained by evolutionary engineering is referred as inverse metabolic engineering (10). Hence, evolutionary engineering has principally a double function (Figure 1): a) it provides a methodology that, together with classical metabolic engineering, has potentials for direct strain improvement in industrial applications; b) it enables the isolation of useful phenotypes for functional and molecular analysis of industrially relevant phenotypes, which can be subsequently reconstructed and transferred in other hosts. Inverse metabolic engineering is the method of choice when apparently simple systems resist straightforward metabolic
engineering, indicating that previously unrecognized factors hamper the improvement of the desired phenotype. Such an example can be found in the implementation of pentose sugar fermentation capacity in yeast for the production of bio-ethanol that is addressed in the following section.
ETHANOL PRODUCTION FROM PENTOSE SUGARS

Starting early in the twentieth century, the petrochemical industry rapidly dominated the market of fuels. Essentially until today, low prices and the illusion of unlimited natural oil reserves triggered a technological development that is heavily dependent on this energy source. The "oil crisis" in the 1970s and the gradually increased environmental pollution level caused by fossil fuel combustion highlighted the importance of finding new alternative energy sources of renewable and less-polluting character.

One low-cost and environmentally compatible alternative is ethanol. Ethanol can be used either alone or as an additive to fossil fuels in conventional combustion engines without major modifications, while maintaining a similar or even increased performance level (112). Being perhaps the oldest product of traditional biotechnology, ethanol is currently produced from fermentation of crop-based substrates (e.g. sugar cane juice or cornstarch), thus is biomass-derived and renewable. The CO₂ released by combustion of bio-ethanol is recycled through natural photosynthetic processes, without a net increase in CO₂ levels. Since increased atmospheric CO₂ is a major cause of global warming by the greenhouse effect, bio-ethanol would provide a basis for sustained energy generation (41). Finally, the addition of ethanol to fossil fuels reduces the emission of carbon monoxide and unburned hydrocarbons that form smog (112). This application has been enforced in recent years (1) and, together with chemical and potable ethanol, increased the global demand for bio-ethanol.

Whether or not ethanol-based fuels have an increasing impact on the market will depend almost exclusively on the production costs because they compete with petroleum-based liquid fuels. Since the cost of raw material for bio-ethanol production may be up to 40% of the product costs (100), research efforts were invested in the establishment of ethanol-producing bioprocesses that start with low-cost raw materials such as lignocellulose. This cheap and abundant polymer mixture is found in agricultural and industrial waste (e.g. wheat straw, corn stalks, sugar cane bagasse, pulp and paper industry waste), but also in forestry residues (109). In general, lignocellulose is constituted of about 45% cellulose, 30% hemicellulose, and 25% lignin (109). While cellulose is completely composed of glucose, hemicellulose contains different hexose (galactose, mannose, rhamnose, fucose) and pentose (xylose, arabinose) sugars, whereas lignin is a macromolecule of phenolic character that cannot be fermented to ethanol in nature. The monomeric constituents of lignocellulose can be released by chemical and/or enzymatic hydrolysis that generates also varying amounts of inhibitory compounds for the fermentation process. These inhibitors include lignin residues, acids and aldehydes such as
phenolic compounds, furaldehydes, acetic, formic and levulinic acid (63). An optimal organism for bioconversion of lignocellulose hydrolysates to ethanol, thus should exhibit a broad substrate utilization range, high ethanol yields and productivities, and high tolerance to ethanol and inhibitors. Ideally, this organism should consume all sugars simultaneously, have the GRAS (Generally Regarded As Safe) status, perform cellulose and hemicellulose hydrolysis by itself, require minimal nutrient supplementation, and exhibit tolerance to low pH and high temperatures. Such an organism does not exist in nature, but a wide variety of possible candidates were evaluated in the past years.

The pentose and hexose fermenting enterobacteria *Escherichia coli* and *Klebsiella oxytoca* have been metabolically engineered to produce ethanol as the major product from lignocellulosic plant material (62). Specifically, expression of pyruvate decarboxylase and alcohol dehydrogenase from *Zymomonas mobilis* sorted the best ethanol yields in *E. coli*, where about 90% of the theoretical yield on sugars could be achieved. Since *E. coli* is a potential pathogen, spent biomass cannot be used directly as fertilizer or animal feed, which is another production cost factor. In addition, the corresponding fermentation process is conducted at neutral pH, and thus particularly sensitive to contaminations.

The GRAS-status bacterium *Z. mobilis* is a high ethanol producer that lacks unfortunately the ability to ferment pentose sugars. The overexpression of xylose and arabinose isomerasers in combination with enzymes of the non-oxidative pentose phosphate pathway, enabled the fermentation of xylose (114) and arabinose (19) and achieved an ethanol yield on pentose sugars of up to about 98% of the theoretical yield. Nevertheless, this organism is sensitive to acetate that is always present in lignocellulose hydrolysates (50).

*Saccharomyces cerevisiae* would be an ideal organism for ethanol production from lignocellulose hydrolysates because of its rare ability among yeasts to grow under anaerobic conditions on glucose (99), high ethanol productivities and yields (51), and inherent tolerance to ethanol and inhibitors present in hydrolysates (48, 64). Unlike bacteria and few yeasts (86), wild type *S. cerevisiae* is not capable of utilizing pentose sugars like xylose and arabinose. Naturally xylose fermenting yeasts such as *Pichia stipitis* (66), *Pachysolen tannophilus* (87), and *Candida shehatae* (66) cannot be used easily, since they are extremely sensitive to metabolic inhibitors (113) and require a well controlled level of aeration for ethanol production (85). Hence, widening the substrate spectrum of *S. cerevisiae* for pentose fermentation has been the subject of intense research in the last decade (12, 33, 38, 69). The particular focus was on xylose, due to its high abundance in lignocellulose.
Xylose fermentation with recombinant *Saccharomyces cerevisiae*

Xylose catabolism in nature follows two distinct pathways. In bacteria, xylose is converted to xylulose by xylose isomerase (XI) (40). In yeasts, xylose is first reduced to xylitol by xylose reductase (XR), which is oxidized to xylulose by xylitol dehydrogenase (XDH) (16) (Figure 2). Although *S. cerevisiae* is not able to grow on xylose (107), some xylose reductase and xylitol dehydrogenase activities were reported (11) and the corresponding genes identified (67, 96). Xylulose is phosphorylated by xylulokinase (XK) to yield xylulose-5-P by a well functioning reaction (74). Xylulose-5-P is then further metabolized via the pentose phosphate pathway. Alternatively, xylulose-5-P may be utilized via a xylose-induced phosphoketolase, which cleaves xylulose-5-P into acetyl-P and glyceraldehyde-3-P (Figure 2). This enzyme occurs typically in lactic bacteria, but was observed in many yeasts, including *S. cerevisiae* (23). Its relevance in yeast central carbon metabolism is unknown, however.

Since natural xylose utilization is too low in wild type *S. cerevisiae*, approaches to establish a high performance xylose catabolism pathway were followed by the expression of a functional xylose isomerase, which does not need any co-factors and should therefore minimize the likelihood of interference with the central metabolic network. While several genes of diverse heterologous origin were expressed without success in *S. cerevisiae* (3, 29, 39, 61, 79), overexpression of the *xylA* gene from *Thermus thermopilosus* yielded an active enzyme, but the activity was still too weak to support growth on xylose (105). Deletion of the endogenous xylose reductase *GRE3* that may produce the xylose isomerase inhibiting compound xylitol (96), and directed evolution of xylose isomerase (56, 57) did not result in higher rates of xylose catabolism. Nevertheless, the recent isolation of a xylose isomerase from the anaerobic fungus *Pirornieis sp.* that was active in *S. cerevisiae* (35) gives some hope for the future establishment of this pathway in yeast.
Figure 2. Xylose catabolic pathways and central carbon metabolism of *S. cerevisiae*. XR: xylose reductase; XDH: xylitol dehydrogenase; XK: xylulokinase; XI: xylose isomerase. The dotted line represents the phosphoketolase reaction and the probable fate of its products.
The first milestone in the integration of a functional xylose catabolic pathway in *S. cerevisiae* was successfully achieved by the expression of the *P. stipitis* xylose reductase and xylitol dehydrogenase (52) (Figure 2). *P. stipitis* XR reduces xylose to xylitol preferentially by oxidizing NADPH (70, 98). In contrast, XDH oxidizes xylitol to xylulose exclusively by reducing NAD⁺ (71). The downside of this approach is the accumulation of xylitol, thus leading to low ethanol productivity and low specific xylose consumption rates (52). Further investigation showed the importance of modulating the activity ratios of XR, XDH and the endogenous XK to optimize ethanol yields and xylose fermentation rates (4, 46, 68, 95, 104). Ethanol yields could be increased at the expense of xylitol accumulation and fermentation rate by partially resolving the intracellular redox balancing problem that results from the different cofactor usage of XR and XDH and from the limited possibilities to re-oxidize NADH anaerobically (44, 73, 102). The xylose fermentation rate was positively correlated with the intracellular concentration of NADPH (6, 44, 73), and with the expression level of xylose reductase (45). Overexpression of the endogenous transaldolase enhanced aerobic growth on sole xylose (106), but simultaneous over-expression of ribulose-5-P epimerase, ribose-5-P isomerase, transaldolase and transketolase did not further improve xylose fermentation, indicating that the non-oxidative pentose phosphate pathway is not limiting under anaerobic conditions yet (47).

Beside the redox balancing problem, xylose transport was thought to be a second main problem for a fast fermentation process. Since specific xylose transporters do not exist in *S. cerevisiae*, xylose is transported via hexose transporters (34). During sole xylose fermentation, however, xylose transport was shown to have only a limited control over the specific xylose utilization rate, unless the xylose metabolism pathway is dramatically improved (30, 34). Since the affinities of these hexose transporters are several folds lower for xylose than for glucose, glucose inhibits xylose transport. This inhibition is a relevant problem when glucose and xylose are simultaneously present, as in lignocellulose hydrolysates. Thus, the expression of a transporter with a high degree of specificity for xylose, which is not inhibited by glucose, would enable the advantageous co-fermentation of xylose and glucose.

**Evolutionary engineering for improved xylose utilization**

Classical deductive metabolic engineering of particular aspects of xylose metabolism (see previous section) and more general regulatory networks (72) has not yet yielded sufficiently high improvements in the anaerobic xylose fermentation efficiency of yeasts, and a
comprehensive understanding of the corresponding bottlenecks is still missing. As a consequence, mutagenesis and selection, as well as evolutionary engineering based strategies have been used increasingly for the isolation of mutants of various yeasts with superior xylose fermenting characteristics (32, 43, 94, 103). In the most recent work, an industrial polyploid S. cerevisiae strain engineered for xylose utilization was subjected to chemical mutagenesis, batch culture selection and finally screened for superior xylose catabolic properties (103). The resulting mutant strain exhibited a five-fold increased aerobic growth rate on xylose and decreased xylitol yields under anaerobic conditions, but no growth on sole xylose under the latter conditions. Molecular analysis of this mutant highlighted the importance of improved xylose transport, increased NADPH production, and increased lower pentose phosphate pathway capacity for improvement of aerobic xylose metabolism in yeast (101). However, the anaerobic xylose fermentation bottlenecks remain still to be investigated.
Chapter 1

METABOLIC ACTIVITY IN THE ABSENCE OF GROWTH

Ideally, a production process has a quick first phase of cell growth, and a second phase during which the cells actively convert substrates into products without diverting any material to further cell growth. Sustained high productivity has a direct impact on the economic competitiveness. Quiescent cells no longer produce biomass and channel their energy and nutritional resources toward the desired production objective. In biotechnological applications of eukaryotes, this goal has often been successfully pursued by controlling the progress of the cells through the cell cycle (27). In these cells, cell cycle arrest in the G1 phase has been induced by nutrient starvation (82), addition of DNA synthesis inhibitors (2), temperature shift (49), metabolic engineering (28, 31), and in some cases resulted in extended protein production phases of up to seven days under non-growing conditions. Compared to eukaryotes, engineering of cell cycle arrest without decreasing productivity appears to be more difficult in bacteria. Growth arrest in E. coli by overexpressing the Rcd RNA (regulator of cell division) in a H-NS (histone-like nucleoid structuring protein) deficient mutant caused growth arrest with, however, only about 10 h of protein production that declined constantly (76). Moreover, the desire to decouple production from growth in bacteria stimulated interest in the corresponding stationary phase response.

As an adaptation to nutrient shortage, which is the prevalent environmental condition in nature, E. coli, like most other microorganisms, has developed a specific strategy for survival in a metabolically inert state (15). The alternative sigma factor $\sigma^5$, encoded by rpoS, plays a central role in modulating gene expression changes upon entry in this starvation-induced state of stationary phase (54). RpoS itself is regulated by other intracellular molecules that are involved in starvation sensing; e. g. ppGpp that is involved in the stringent response (37) and the carbon catabolite repression modulating cAMP (54). Since the regulation of rpoS takes place at the transcriptional, translational, and post translational level (54), the control of stationary phase appears to be extremely complicated and a comprehensive model is not yet available. In addition, different regulatory systems have been shown to be active depending on which nutrient is limiting: carbon, nitrogen or phosphate (24).

The biotechnologically most relevant consequence of stationary phase induction in E. coli is a dramatic decrease in metabolic rates. The relief of this phenomenon is essential to achieve decoupling of production from growth and increased operational process live-spans. Classical metabolic engineering strategies are not straightforward because essential insight in the corresponding regulatory network is still lacking. Evolutionary engineering, in contrast,
suffers from the apparent paradox of selecting a growth unrelated property with procedures which favor improved growth characteristics.
Chapter 1

OUTLINE OF THE THESIS

In this thesis, I address evolutionary engineering of two biotechnologically relevant phenotypes that were difficult to achieve by rational metabolic engineering because of the absence of sufficient functional information: a) the improvement of anaerobic xylose catabolism in *S. cerevisiae* and b) the increase of metabolic activity in non-growing *E. coli*.

In **Chapter 2**, we isolated and characterized the first yeast able to grow on sole xylose under strict anaerobic conditions by a 460 generation chemostat-based selection procedure. This unique yeast strain was then investigated in great detail by combining global gene expression, and intracellular metabolic flux analysis to elucidate the enabling molecular characteristics in **Chapter 3**. Potential limitations in the pentose phosphate pathway, redox balancing, and xylose transport were investigated in **Chapter 4** by several specific evolution experiments and by expressing the *E. coli* transhydrogenase UdhA in recombinant, xylose-fermenting *S. cerevisiae*.

Using a rational metabolic engineering approach, a new pathway for NADH re-oxidation in xylose-fermenting *S. cerevisiae* was installed by overexpressing two enzymes of the phosphoketolase pathway in **Chapter 5**.

**Chapter 6** quantitatively compares the fermentation performance and robustness of several recently developed strains of laboratory and industrial origin.

Finally, in **Chapter 7**, we identified a selection scheme that favors *E. coli* mutants with increased catabolic rates in stationary phase, yielding mutants with doubled rates after only 40 generations.
REFERENCES


27


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Evolutionary engineering of *Saccharomyces cerevisiae* for anaerobic growth on xylose

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SUMMARY

Xylose utilization is of commercial interest for efficient conversion of abundant plant material to ethanol. Perhaps the most important ethanol producing organism, *Saccharomyces cerevisiae*, however, is incapable of xylose utilization. While *S. cerevisiae* strains were metabolically engineered to utilize xylose, none of these recombinant strains, nor any other naturally occurring yeast, were able to grow anaerobically on xylose. Starting with the recombinant *S. cerevisiae* strain TMB3001 that overexpresses the xylose-utilization pathway from *Pichia stipitis*, we describe here a selection procedure for the evolution of strains that are capable of anaerobic growth on sole xylose. Selection was only successful when TMB3001 was first selected for efficient aerobic growth on sole xylose and then slowly adapted to microaerobic and finally anaerobic conditions, indicating that multiple mutations were necessary. After a total of 460 generations or 266 days of selection, the culture propagated stably under anaerobic conditions on xylose and consisted primarily of two subpopulations with distinct phenotypes. Clones of the larger subpopulation grew anaerobically on xylose, co-utilized xylose and glucose in batch culture, but exhibited impaired growth on glucose. Surprisingly, clones of the smaller subpopulation were incapable of anaerobic growth on xylose. However, as a consequence of their improved xylose catabolism, these clones produced up to 19% more ethanol, when compared to the parental TMB3001 under process-like conditions from a mixture of glucose and xylose.
INTRODUCTION

Over the last decade, metabolic engineering became a standard for strain improvement and has been very successful when simple cellular traits were targeted (20, 27). Although the genomics age with the associated genome-wide analytical technologies gives further impetus to rational approaches, metabolic engineering of more complex or not fully understood cellular systems remains a challenge (4). Akin to random, combinatorial approaches such as directed evolution in contemporary protein engineering (3), evolutionary approaches are becoming increasingly important to augment metabolic engineering of complex phenotypes (22, 23, 25, 37). In certain cases, however, even seemingly simple metabolic systems resist straightforward rational engineering.

One example of a seemingly simple trait is expanding the substrate range of *Saccharomyces cerevisiae* for the utilization of pentoses for ethanol formation. The commercial interest in pentose utilization, in particular xylose, is related to the prevalence of pentoses in abundant plant material, for instance as the major structural unit in hemicelluloses. While metabolic engineering has successfully endowed *S. cerevisiae* with the ability to utilize the pentoses xylose (8, 13, 18, 34) and recently also arabinose (5), it has not yet succeeded in developing strains that convert pentose at a high yield and a high specific rate to ethanol (11, 14).

An additional puzzling fact is the inability of xylose to support anaerobic growth in both natural and recombinant xylose-utilizing yeasts (17). Since many bacteria can grow anaerobically on xylose (15), the reason for this inability is not really understood at present, but has been ascribed to a general restriction of eukaryotic xylose metabolism to respirative conditions (17). This argument was based on the fundamental difference between eukaryotic and prokaryotic xylose catabolism because bacteria convert xylose directly to xylulose using xylose isomerase, whereas eukaryotes generally rely on two consecutive redox reactions that are catalyzed by the NADPH-dependent xylose reductase (XR) and the NADH-dependent xylitol dehydrogenase (XDH) with xylitol as the pathway intermediate. By providing NADPH through the oxidative pentose phosphate pathway, which operates actively in *S. cerevisiae* and *Pichia stipitis* (9, 10), and by respiring NADH, eukaryotes can efficiently drive these coupled redox reactions under aerobic conditions, but possibly not under anaerobic conditions. While this could potentially explain the inability of many yeasts to grow anaerobically on xylose, it does not suffice as an explanation for those xylose-utilizing *S. cerevisiae* strains that
functionally overexpress xylose isomerase (33). Hence, it appears that at least one additional component in our understanding of xylose metabolism is missing.

Such understanding cannot be obtained from the available databases and the published knowledge, hence we attempt here to evolve strains that are capable of anaerobic growth on xylose by long-term selection experiments. Since anaerobic growth on xylose did apparently not evolve naturally in yeast, selection should be initiated with the best xylose-utilizing S. cerevisiae strains available. The presently best strains overexpress the XR and XDH genes from P. stipitis (18) in combination with the endogenous xylulokinase (XK) (8, 13, 29). We used the S. cerevisiae strain TMB3001 that overexpresses the three genes of the xylose-utilization pathway from a chromosomal integration to initiate various long-term evolution experiments (8).
MATERIALS AND METHODS

Strains and media

All evolution experiments were inoculated with the recombinant *S. cerevisiae* strain TMB3001 (CEN.PK 113-7A (MATα, his3::Δ1, MAL2-8c, SUC2) his3::YIpXR/XDH/XK), which contains the entire xylose-utilization pathway (8). Overexpression of XR is controlled by the alcohol dehydrogenase promoter and terminator, whereas XDH and XK are both under the control of phosphoglycerate kinase promoters and terminators. The extensions C1 to C15 refer to clones that were isolated after 460 generations of selection. TMB3001C1 and TMB3001C5 are representatives of the phenotypic classes II and I, respectively.

For physiological analysis and evolution experiments, yeast cultures were grown at 30°C in minimal medium containing per litre: 5 g (NH₄)₂SO₄, 3 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 15 mg EDTA, 4.5 mg ZnSO₄·7H₂O, 0.3 mg CoCl₂·6H₂O, 1 mg MnCl₂·4H₂O, 0.3 mg CuSO₄·4H₂O, 4.5 mg CaCl₂·2H₂O, 3 mg FeSO₄·7H₂O, 0.4 mg Na₂MoO₄·2H₂O, 1 mg H₃BO₃, 0.1 mg KI, 0.05 mg biotin, 1 mg Ca pantothenate, 1 mg nicotinic acid, 25 mg inositol, 1 mg thiamine HCl, 1 mg pyridoxine HCl, and 0.2 mg para-aminobenzoic acid (pH 5.0) (31). In chemostat cultures, 0.1 g l⁻¹ polypropylene glycol P 2000 was added to prevent foam formation. The medium was supplemented with ergosterol (Fluka) and Tween 80 (Sigma) for anaerobic cultivation. Both components were dissolved in boiling 99.8% (v/v) ethanol and were added to the medium at a final concentration of 0.01 g l⁻¹ and 0.42 g l⁻¹, respectively. Solid media were prepared by adding 1.5% (w/v) technical Agar (Becton Dickinson). For anaerobic growth on xylose plates, population aliquots were washed twice with PBS (8 g l⁻¹ NaCl, 0.2 g l⁻¹ KCl, 1.44 g l⁻¹ Na₂HPO₄, 0.24 g l⁻¹ KH₂PO₄, pH 7.0) and plated on anaerobic minimal medium containing 20 g l⁻¹ xylose as the sole carbon source. Plates were incubated at 30°C in sealed jars, using the GasPack Plus system (Becton Dickinson) to provide an anaerobic atmosphere, which was verified by indicator strips (Becton Dickinson).

Long-term selection cultures

Chemostat selections were performed in a Sixfors 6-minireactors system (Infors, Botmingen, Switzerland) at a dilution rate (D) of 0.05 h⁻¹ and mixing at 300 rpm. A constant working volume of 300 ml was maintained by continuously removing excess culture broth through a needle that was fixed at a predetermined height. The culture pH was maintained at 5.0 ± 0.3 by supplementing the minimal medium with 50 mM potassium hydrogen phthalate.
(Fluka) (32). Aerobic conditions were installed by aeration at a rate of 0.3 l min\(^{-1}\). Microaerobic conditions were installed by stepwise reduction of aeration until no measurable flow was seen in the reactor effluent gas. Anaerobic conditions were established by slight sparging (< 1 ml min\(^{-1}\)) with technical N\(_2\) (< 200 ppm O\(_2\); independently quantified with a Prima 600 mass spectrometer (Fisons Instruments, Uxbridge, England)). It should be noted that due to the contaminating O\(_2\), these conditions are not strictly anaerobic. To ensure robust long-term operation of up to 4 months, marprene tubing (Ismatech, Glattbrugg, Switzerland) was used with external peristaltic pumps for feeding and harvesting. Contamination controls were done in two week intervals by plating culture aliquots on YPD medium (10 g l\(^{-1}\) yeast extract, 20 g l\(^{-1}\) peptone, and 20 g l\(^{-1}\) glucose) plates and by microscopic analysis.

Selection in serial, strict anaerobic batch cultures was done in Hungate tubes, which are 17 ml Pyrex glass tubes that are sealed with butyl rubber septa and plastic screw caps (Bellco Glass Inc., Vineland, NJ). Cultures were grown in minimal medium, containing 10 g l\(^{-1}\) xylose as the sole carbon source. New cultures were inoculated when the growth rate declined, which occurred typically after about 1 week.

**Growth conditions**

Aerobic cultures were grown in 500 ml baffled shake flasks with 50 ml minimal medium at 300 rpm on a rotary shaker and 30°C. To adapt TMB3001 to aerobic growth on sole xylose, it was first grown on YPX medium (10 g l\(^{-1}\) yeast extract, 20 g l\(^{-1}\) peptone, and 20 g l\(^{-1}\) D-xylose), then on YNB xylose medium (6.7 g l\(^{-1}\) Yeast Nitrogen Base and 20 g l\(^{-1}\) D-xylose), and finally once in minimal medium with sole xylose prior to inoculation.

Fermentation performance was evaluated in anaerobic batch cultures containing 50 g l\(^{-1}\) each of glucose and xylose. The concentrations of all other minimal medium components except KH\(_2\)PO\(_4\) were doubled. To avoid major drops in pH, 100 mM citric acid buffer (pH 5.5) was added, which maintained the pH above 4.7 in all cases. Cultures were grown in 175 ml serum bottles, filled with 150 ml medium and stirred magnetically at 100 rpm and 30°C. Anaerobic (but not strictly anaerobic) conditions were maintained by slight continuous sparging (1-2 bubbles sec\(^{-1}\)) with technical N\(_2\) (O\(_2\) < 200 ppm) (PanGas, Dagmersellen, Switzerland). Inocula were prepared by growing frozen stock cultures first on YPD medium and finally in minimal medium with 20 g l\(^{-1}\) glucose.

Strictly anaerobic growth experiments on sole xylose were done in Hungate tubes or serum bottles sealed with butyl-rubber septa by sparging the basic salt solution of the minimal medium with pure N\(_2\) (O\(_2\) < 5 ppm) (PanGas) for 15 min. After autoclaving, the
remaining filter-sterilized, N₂-sparged medium components and 10 g l⁻¹ xylose were added. To ensure strict anaerobiosis, 0.25 g l⁻¹ Na₂S or 0.5 g l⁻¹ L-cysteine were added in selected cases as reducing agents after incubation at 60°C for 5 min. Withdrawal of culture aliquots was done under purging with pure N₂. To verify strict anaerobiosis, the redox indicator resazurin was added to the medium at a final concentration of 0.0001% (w/v) before sparging with pure N₂. Strict anaerobic growth experiments on sole xylose were inoculated with cultures grown on 20 g l⁻¹ glucose minimal medium. Inocula were washed twice with PBS prior to inoculation to avoid glucose contamination.

Stocks for strain maintenance were generated from overnight cultures grown in YPD medium by adding glycerol to a final concentration of 15% (w/v) and were then stored at −80°C. To preserve the original clonal composition of selection chemostats, population aliquots from the selection cultures were frozen directly, without intermediate batch growth.

EMS mutagenesis

To increase genetic variability, cultures were randomly mutagenized with ethyl methane sulfonate (EMS) (Sigma), by harvesting population aliquots at 1,500 rpm and 4°C for 3 min from minimal medium batch cultures in early stationary phase. Pellets were washed once with PBS and resuspended in 10 ml PBS. 300 µl of EMS were added and the suspension was incubated on a rotatory shaker at 300 rpm and 30°C. After 40 min, 20 ml 5% (w/v) Na₂S₂O₃ were added to inactivate the mutagen. After centrifugation, the pellet was washed twice with 5% (w/v) Na₂S₂O₃ to remove residual EMS, resuspended in 20 ml minimal medium, and stored at −80°C after addition of 15% (w/v) glycerol. Survival rates of 5 to 30% were verified by counting colony-forming units.

Analytical methods

Cell growth was monitored by following the optical density at 600 nm (OD₆₀₀) or by determining the Klett-value with a Klettmeter (Bel-Art Products, Pequonock, NJ). Cellular dry weight (cdw) was determined from 10 ml culture aliquots that were centrifuged at 5,000 rpm for 20 min in pre-weighed glass tubes, washed once with water, and dried at 110°C for 24 h to constant weight. Commercially available kits were used for enzymatic determination of glucose (Beckman), xylose (Medichem, Steinenbronn, Germany), xylitol (R-Biopharm, Darmstadt, Germany), acetate (R-Biopharm), and glycerol (Sigma). Ethanol concentrations
were determined by gas chromatography (5890E chromatograph; Hewlett-Packard) with a Permabond-CW20M-0.25 column (Macherey-Nagel) and butyrate as the internal standard.

**Determination of physiological parameters**

In batch culture, exponential growth rates were determined by log-linear regression of OD<sub>600</sub> versus time with growth rate (μ) as the regression coefficient. The specific biomass yield (Y<sub>X/S</sub>) was determined as the coefficient of linear regression of biomass concentration (X) versus substrate concentration (S) during the exponential growth phase. The biomass concentration was estimated from predetermined OD<sub>600</sub>-to-cdw correlations during the mid-exponential growth phase of aerobic cultures on glucose for TMB3001, TMB3001C5, and TMB3001C1 (0.530, 0.581, and 0.479 g OD<sub>600</sub>⁻¹, respectively). During the exponential growth phase, specific glucose or xylose uptake rates (q<sub>glc</sub> or q<sub>xyl</sub>) were calculated as the ratio between μ and Y<sub>X/S</sub>. Ethanol, xylitol, acetate, and glycerol yields were calculated by linear regression of by-product concentration versus S.

In mixed substrate fermentation analysis, the specific xylose uptake rate was determined as the ratio of the linear regression coefficient of xylose concentration versus time and the average biomass concentration between the onset of xylose consumption and about 100 h after inoculation. In these cases, the OD<sub>600</sub>-to-cdw correlation was determined at the end of each fermentation.
RESULTS

Chemostat selection

Evolution of a yeast strain capable of anaerobic growth on sole xylose was started with the metabolically engineered *S. cerevisiae* strain TMB3001, which overexpresses XR and XDH from *P. stipitis* and the native XK from a stable chromosomal insertion (8). To increase genetic variability, an EMS-mutagenized population of TMB3001 was used to inoculate all selection procedures. Direct gain-of-function selections for growth on sole xylose in batch cultures or in petri-dishes proved to be unsuccessful under anaerobic conditions (data not shown). Similarly, extended selection in serial anaerobic batch cultures with xylose in combination with glucose for more than 30 generations did not yield a population capable of anaerobic growth on sole xylose (data not shown). Hence, we initiated two long-term anaerobic chemostats at a D of 0.05 h\(^{-1}\) that contained a limiting concentration (1 g l\(^{-1}\)) of the growth-promoting sugars glucose or galactose and 5 g l\(^{-1}\) of xylose. Galactose was chosen to avoid catabolite repression and competitive inhibition of xylose transport by glucose (11). Within about 170 generations (100 days), the steady-state biomass concentrations remained unaltered and only 5 to 10% of the supplied xylose were consumed in both cultures (data not shown), indicating the absence of any evolutionary progress toward anaerobic growth on xylose.

To facilitate sequential evolution of multi-gene changes that may be required for efficient anaerobic xylose catabolism, an aerobic chemostat culture with 5 g l\(^{-1}\) xylose and 1 g l\(^{-1}\) glucose was initiated (Figure 1A). After about 30 generations, the steady-state xylose concentration declined and OD\(_{600}\) increased. Within 90 generations a new steady-state was attained, during which 80% of the supplied xylose was consumed. At this stage, a culture aliquot was withdrawn, EMS-mutagenized, and used to inoculate two new aerobic chemostat cultures. Settings in the first chemostat were identical to the previous one and a comparable steady-state was attained immediately. This chemostat was then switched to anaerobiosis and OD\(_{600}\) decreased to 0.4 while the residual xylose concentration increased to 4.5 g l\(^{-1}\) (data not shown). This steady-state physiology was similar to the previous direct anaerobic selection on 5 g l\(^{-1}\) xylose and 1 g l\(^{-1}\) glucose. Since no significant improvements were observed during the following 30 generations, this culture was not followed further. The second, aerobic chemostat contained 5 g l\(^{-1}\) xylose as the sole carbon source (Figure 1B). Different from the initial EMS-mutagenized TMB3001 population, we obtained a growing population, which
consumed increasingly more xylose, thereby decreasing the residual xylose concentration from 1.5 g l\(^{-1}\) to 0.3 g l\(^{-1}\) after 60 generations. To install micro-aerobic conditions, the aeration rate was drastically reduced from 0.3 l min\(^{-1}\) to below 1 ml min\(^{-1}\) at generation 140. Within the following 20 generations, the residual xylose concentration increased and OD\(_{600}\) decreased rapidly. When OD\(_{600}\) appeared to be stable, aeration was turned off at generation 170. After an immediate rise, the residual xylose concentration decreased and OD\(_{600}\) increased steadily for 100 generations (Figure 1B). At a residual xylose concentration of 0.4 g l\(^{-1}\) and an OD\(_{600}\) of 3.1 at generation 270, anaerobic conditions were established by continuous sparging with technical N\(_2\). Soon after the onset of anaerobiosis, a stable steady-state was attained, albeit with a low OD\(_{600}\) (Figure 1B). To elucidate whether anaerobic growth on xylose could be improved further, we EMS mutagenized an aliquot withdrawn at generation 310. Upon anaerobic batch growth on xylose as the sole carbon source, an anaerobic chemostat culture was grown for another 150 generations on xylose, during which a gradual increase in biomass formation was observed (Figure 1C). It should be noted that due to an O\(_2\) contamination of below 200 ppm in the N\(_2\), conditions were not strictly anaerobic in this selection culture, as will also be shown later.

To identify the time point at which the capability for anaerobic growth on sole xylose emerged first in the 460 generations (266 days) of evolution, frozen aliquots from generations were plated on xylose minimal medium and incubated in a strictly anaerobic atmosphere. Colonies were only detected in population aliquots after 270 generations. To exclude the possibility that anaerobic growth of the evolved population was due to trace amounts of O\(_2\) in the technical N\(_2\), the final evolved population was grown in batch cultures with increasing strengths of anaerobiosis. When anaerobiosis was established in serum bottles by continuous sparging with technical N\(_2\) (O\(_2\) < 200 ppm), the maximum biomass concentration was 0.50 ± 0.08 g\(_{\text{cdw}}\) l\(^{-1}\) on 5 g l\(^{-1}\) xylose. When anaerobiosis was established in tightly sealed, anaerobic Hungate tubes (without continuous N\(_2\) sparging), however, the maximum biomass concentration was only 0.24 ± 0.03 g\(_{\text{cdw}}\) l\(^{-1}\) on 10 g l\(^{-1}\) xylose. This lower biomass yield under more stringent anaerobiosis clearly demonstrates the physiologically relevant role of contaminating O\(_2\); not only in these batch cultures but likely also during the anaerobic selection. Finally, anaerobic growth on sole xylose in Hungate tubes with the addition of Na\(_2\)S or cysteine as reducing agents and resazurin as a redox indicator confirmed the gain-of-function phenotype (data not shown). In all cases xylose was completely consumed at the end of growth.
Figure 1. Evolution of *S. cerevisiae* TMB3001 in carbon-limited chemostat cultures at D of 0.05 h\(^{-1}\) under aerobic conditions with 5 g l\(^{-1}\) xylose and 1 g l\(^{-1}\) glucose (A); aerobic, microaerobic (light gray background), and anaerobic (dark gray background) conditions with 5 g l\(^{-1}\) xylose (B); and anaerobic conditions with 5 g l\(^{-1}\) xylose (C). (1) Airflow reduction from 0.3 l min\(^{-1}\) to < 1 ml min\(^{-1}\), (2) shut-off of airflow, and (3) onset of anaerobiosis by sparging with technical N\(_2\). The evolving population was subjected to EMS mutagenesis prior to inoculation of the chemostats A, B, and C.

**Clonal analysis**

Since chemostat-evolved, asexual populations are typically heterogeneous (19, 24, 35), a population aliquot after 460 generations was plated on xylose minimal medium. Under anaerobic conditions the number of colony-forming units was 54 ± 4% of that seen on aerobic YPD plates, thus providing first evidence for population heterogeneity. The parental strain TMB3001, the evolved population at 460 generations, and 15 clones, which were isolated
from anaerobic xylose plates, were then compared for their fermentation performance in anaerobic batch cultures with 50 g l$^{-1}$ glucose and 50 g l$^{-1}$ xylose. During the initial phase of exponential growth on glucose, almost no xylose was consumed but upon glucose depletion, growth ceased and xylose was consumed in a second phase (Figure 2).

![Fermentation profile](image-url)

**Figure 2.** Fermentation profile of TMB3001 (A), the 460-generation selection (B), clone TMB3001C5 representing the first phenotypic class (C), and clone TMB3001C1 representing the second phenotypic class (D), during anaerobic growth on 50 g l$^{-1}$ glucose and 50 g l$^{-1}$ xylose. Glucose and xylose consumption phases are highlighted by I and II, respectively. Gray shading indicates simultaneous consumption of glucose and xylose.
Figure 3. Physiological parameters during anaerobic growth on 50 g l⁻¹ glucose and 50 g l⁻¹ xylose of TMB3001, the 460-generation population, and 15 clones isolated from this population. Maximum growth rate and biomass yield were determined during exponential growth on glucose (A). Specific xylose uptake rate and xylitol yield on xylose were determined between glucose depletion and 100 h of fermentation (B). The final ethanol concentration was determined at 180 h (C). Values for TMB3001 and the population are average values from duplicate experiments. Hairlines indicate the reference values of TMB3001.
To quantitatively compare clonal fermentation performance, physiological parameters were determined during the glucose and the xylose consumption phases (Figure 3). Since the specific xylose uptake decrease after about 100 h of fermentation (Figure 2), parameters for the second phase were calculated for the period between glucose depletion and 100 h. Generally, two major phenotypic classes could be discerned. The first class of clones and the evolved population were similar to TMB3001 during growth on glucose, but exhibited on average an about 60% higher specific xylose uptake rate and reduced formation of the by-product xylitol during the second phase (Figures 2A, B, C and 3). As a consequence, these clones accumulated up to 19% higher final ethanol concentrations (Figure 3C). The more abundant second class of clones exhibited a radically different mode of growth on glucose (Figures 2D and 3). On average, the maximum growth rate and biomass yield on glucose were reduced by 60% and 40%, respectively. Unlike TMB3001 and the class I clones, the class II clones began to consume xylose prior to glucose depletion (Figure 2D). The specific xylose uptake rates were at least doubled in the class II clones, ranging from \(0.19 \text{ g g}^{-1} \text{ h}^{-1}\) (TMB3001C12) to \(0.31 \text{ g g}^{-1} \text{ h}^{-1}\) (TMB3001C1), when compared to \(0.08 \text{ g g}^{-1} \text{ h}^{-1}\) for TMB3001. Xylitol yields (Figure 3B) but also final ethanol accumulation (Figure 3C) were reduced in these clones, when compared to TMB3001.

Determination of the time course of acetate and glycerol for selected clones of both classes confirmed the drastic physiological changes of the class II phenotype (Figure 4). Compared to TMB3001 and the class I clones, the class II clones produced significantly more acetate and glycerol on both glucose and xylose.
Figure 4. Yields of acetate (A) and glycerol (B) on glucose (black bars) and xylose (open bars) during anaerobic growth on 50 g l\(^{-1}\) glucose and 50 g l\(^{-1}\) xylose of TMB3001 and selected clones from both phenotypic classes. Yields on glucose were determined between inoculation and the begin of xylose uptake. Yields on xylose were determined between glucose depletion and 130 h. Values were determined from single experiments.

Physiological characterization

To further elucidate the phenotypic differences of the two co-evolved subpopulations, the best representatives of each phenotypic class were grown in single substrate batch cultures. The class II representative TMB3001C1 exhibited significantly slower aerobic glucose catabolism with a 36% reduced growth rate and a 48% reduced specific rate of glucose uptake, when compared to TMB3001 (Table 1). Although the efficiency of exponential growth on glucose was not affected, as judged from the maximum biomass yield (Y\(_{X/S}\)), the maximum biomass concentration attained by TMB3001C1 was significantly lower.
### TABLE 1. Physiological parameters of TMB3001, TMB3001C5 (class I), and TMB3001C1 (class II) in aerobic batch culture with 5 g l⁻¹ glucose.

<table>
<thead>
<tr>
<th>Strain</th>
<th>$\mu_{\text{max}}$ (h⁻¹)</th>
<th>$q_{\text{glc}}$ (g g⁻¹ h⁻¹)</th>
<th>$Y_{X/S}$ (g g⁻¹)</th>
<th>Biomass$_{\text{max}}$ (g l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMB3001</td>
<td>0.44ᵃ</td>
<td>3.14 ± 0.05</td>
<td>0.14ᵇ</td>
<td>2.1ᶜ</td>
</tr>
<tr>
<td>TMB3001C5</td>
<td>0.41</td>
<td>2.61 ± 0.09</td>
<td>0.16</td>
<td>2.2</td>
</tr>
<tr>
<td>TMB3001C1</td>
<td>0.28</td>
<td>1.62 ± 0.02</td>
<td>0.17</td>
<td>1.4</td>
</tr>
</tbody>
</table>

ᵃDeviation from duplicate experiments, ± 0.01
ᵇDeviation, ± 0.01.
ᶜDeviation, ± 0.02.

### TABLE 2. Physiological parameters of TMB3001, TMB3001C5 (class I), and TMB3001C1 (class II) in aerobic batch culture with 5 g l⁻¹ xylose.

<table>
<thead>
<tr>
<th>Strain</th>
<th>$\mu_{\text{max}}$ (h⁻¹)</th>
<th>$q_{\text{xyl}}$ (g g⁻¹ h⁻¹)</th>
<th>$Y_{X/S}$ (g g⁻¹)</th>
<th>Biomass$_{\text{max}}$ (g l⁻¹)</th>
</tr>
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<tbody>
<tr>
<td>TMB3001</td>
<td>0.016ᵇᵇ</td>
<td>n. d.ᶜ</td>
<td>n. d.</td>
<td>2.1ᵈ</td>
</tr>
<tr>
<td>TMB3001C5</td>
<td>0.064</td>
<td>0.13 ± 0.00</td>
<td>0.50 ± 0.02</td>
<td>1.9</td>
</tr>
<tr>
<td>TMB3001C1</td>
<td>0.119</td>
<td>0.27 ± 0.02</td>
<td>0.45 ± 0.04</td>
<td>2.0</td>
</tr>
</tbody>
</table>

ᵃAerobic growth on sole xylose was observed only after serial growth in YPX and YNB medium with 20 g l⁻¹ xylose.
ᵇD from duplicate experiments, ± 0.001.
ᶜNot determined.
ᵈDeviation, ± 0.02

During aerobic growth on sole xylose, both clones grew significantly faster than their parent, but TMB3001C1 grew by far the most rapid (Table 2). Surprisingly, only TMB3001C1 but not TMB3001C5 was capable of strict anaerobic growth on sole xylose in Hungate tubes (Figure 5). Further increased strength of anaerobiosis by addition of cysteine (Table 3) or Na₂S (data not shown) had no significant impact on the growth of TMB3001C1.
Compared to the evolved population, which also includes this clones, TMB3001C1 grew significantly faster on xylose under anaerobic conditions.

![Graph showing OD600 and xylose concentration during strict anaerobic growth of TMB3001C1 in minimal medium with xylose as sole carbon source.](image)

**Figure 5.** OD<sub>600</sub> and xylose concentration during strict anaerobic growth of TMB3001C1 in minimal medium with xylose as sole carbon source.

To elucidate the reason for the persistence of the class I clones in the evolved population, despite their inability to grow anaerobically on sole xylose, we established anaerobic conditions akin to those in the final selection chemostat by continuous sparging (<1 ml min<sup>-1</sup>) with technical N<sub>2</sub> (<200 ppm O<sub>2</sub>). Surprisingly, all three strains, TMB3001, TMB3001C1, and TMB3001C5, grew to an OD<sub>600</sub> of about 0.2 on minimal medium without carbon source supplementation, presumably on the 0.5 g l<sup>-1</sup> of ethanol that was added with the ergosterol and Tween 80 stock solution, and which provides sufficient carbon for the observed growth. Since neither TMB3001 nor TMB3001C5 grew on xylose under these not strictly anaerobic conditions, it appears that the class I clones survived in the selection by scavenging contaminating O<sub>2</sub> for oxidation of the produced ethanol or possibly other metabolic by-products of the class II clones. The growth rate of TMB3001C1 on xylose was 0.07 h<sup>-1</sup> under these conditions, compared to 0.012 h<sup>-1</sup> under strict anaerobic conditions (Table 3). This indicates that also TMB3001C1 benefited from O<sub>2</sub> contamination and explains why these class II clones are not washed out in the anaerobic chemostat at a D of 0.05 h<sup>-1</sup>.
TABLE 3. Physiological parameters of TMB3001C1 and the 460-generation population in strict anaerobic batch culture with 10 g l⁻¹ xylose.

<table>
<thead>
<tr>
<th>Strain</th>
<th>( \mu_{\text{max}} ) (h⁻¹)</th>
<th>( q_{\text{xyl}} ) (g g⁻¹ h⁻¹)</th>
<th>Biomass (g g⁻¹)</th>
<th>Ethanol (g g⁻¹)</th>
<th>Xylitol (g g⁻¹)</th>
<th>Glycerol (g g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMB3001C1</td>
<td>0.012b</td>
<td>0.56c</td>
<td>0.021 ± 0.004</td>
<td>0.24d</td>
<td>0.32 ± 0.00</td>
<td>0.044 ± 0.005</td>
</tr>
<tr>
<td>TMB3001C1 + Cysc</td>
<td>0.010</td>
<td>0.52</td>
<td>0.022 ± 0.008</td>
<td>0.21</td>
<td>0.37 ± 0.09</td>
<td>0.047 ± 0.008</td>
</tr>
<tr>
<td>460-gen. population</td>
<td>0.004</td>
<td>0.23</td>
<td>0.018 ± 0.006</td>
<td>0.25</td>
<td>0.33 ± 0.01</td>
<td>0.036 ± 0.001</td>
</tr>
</tbody>
</table>

\(^{a}\) Acetate yields were below 0.006 g g⁻¹ in all cases.
\(^{b}\) Deviation from duplicate experiments, ± 0.001.
\(^{c}\) Deviation, ± 0.15.
\(^{d}\) Deviation, ± 0.01.
\(^{e}\) Anaerobiosis was installed by addition of cystein.

**Batch culture selection**

Since strict anaerobic growth on xylose of the best isolated clone was still relatively slow, an EMS-mutagenized population of TMB3001C1 was grown sequentially in seven serial batch cultures, which corresponds to 40 generations. 20 isolated clones were then grown on sole xylose in strict anaerobic Hungate tubes (Figure 6). Relative to the maximum growth rate of TMB3001C1, seven clones grew at about the same rate, ten clones grew 1.2- to 2-fold faster, and three clones grew 2- to 2.5-fold faster. The highest growth rate observed was 0.028 h⁻¹ for the clones 1 and 18. The three best clones were then characterized more accurately in anaerobic xylose batch cultures. While TMB3001C1 grew at the previously determined rate of 0.012 h⁻¹, clones 1, 14, and 18 grew at 0.027 ± 0.002 h⁻¹, 0.021 ± 0.002 h⁻¹, and 0.018 ± 0.002 h⁻¹, respectively.
Figure 6. Strict anaerobic growth rates on xylose minimal medium of 20 clones that were isolated after seven serial anaerobic batch cultures on xylose. The hairline indicates the growth rate of the parental TMB3001C1 before selection.
DISCUSSION

We describe here to our knowledge the first yeast strain that grows on xylose as the sole carbon source under strict anaerobic conditions. Such strains were isolated from a long-term, multi-step chemostat evolution experiment, which was initiated with the metabolically engineered *S. cerevisiae* strain TMB3001 that overexpresses the xylose-utilization pathway of *P. stipitis* (8). The selection procedure was based on the well-known evolution of mutants with increased substrate affinity and utilization in chemostat cultures (1, 7, 25, 26, 35). However, the key to successful evolution was to decouple selection for aerobic and anaerobic xylose utilization (Figure 1). Thus, the selective pressure was adjusted to the present capabilities of the evolving culture, allowing advantageous mutations to accumulate under growth permissive conditions.

Although the clones described here were isolated after 460 generations or 266 days of selection, the ability to grow anaerobically on sole xylose was first detected after 270 generations, immediately after switching the culture conditions to anaerobiosis (< 200 ppm O₂). The achieved phenotype of the best xylose-utilizing clone TMB3001C1, with a maximum specific growth rate of 0.012 h⁻¹ and a biomass yield of 0.021 g g⁻¹ during strict anaerobic growth on xylose, represents by no means a final stage of evolution. For instance, the anaerobic growth rate of TMB3001C1 could be more than doubled within 40 generations of batch culture selection (Figure 6). Although the rate of anaerobic xylose metabolism is still relatively slow, the isolation of these improved clones argues against the view that eukaryotic xylose metabolism is necessarily tied to respiration (17). Our results are more consistent with the view that anaerobic growth on xylose does not naturally occur in yeasts because the rate of xylose metabolism is too slow, so that the rate of ATP production is insufficient (11, 18). Since the strains evolved here consume xylose at a several-fold higher specific rate than for example the control strain TMB3001, one would expect that the accumulated beneficial mutations affect, at least in part, the rate of catabolism and thus ATP formation. The nature of the underlying genetic changes that cause the observed phenotypic changes remains unclear at present and are subject to further investigation in our lab. Multiple mutations were probably necessary to endow TMB3001 with the capability of strict anaerobic growth on xylose, since direct selection on plate, in batch, or chemostat culture failed. This may also explain to some extend why intense rational metabolic engineering efforts have not yet yielded such strains (2, 11, 14).
After 460 generations, the population consisted of at least two subpopulations with distinct phenotypes, thus evidencing population heterogeneity (or polymorphism) that is often observed during evolution experiments (12, 19, 24, 30, 35). The class I phenotype of the smaller subpopulation, representing one third of the isolated clones, was rather similar to the parental TMB3001 on glucose but was significantly improved on xylose. The best representative of these, TMB3001C5, exhibited a 60% higher specific xylose uptake rate and a four-fold higher aerobic growth rate on sole xylose, when compared to TMB3001 (Table 2). Consequently, this strain accumulated up to 19% more ethanol when grown anaerobically under process-like conditions in a mixture of 50 g l\(^{-1}\) each of glucose and xylose (Figure 3C). None of these class I clones, however, grew anaerobically on sole xylose; neither under strict anaerobiosis nor in the presence of contaminating O\(_2\). The class II phenotype of the more abundant subpopulation was characterized by an even further improved xylose metabolism and was additionally capable of strict anaerobic growth on xylose. The best representative of this subpopulation, TMB3001C1, exhibited a more than three-fold higher specific xylose uptake rate and an eight-fold higher aerobic growth rate on xylose, when compared to TMB3001 (Table 2). All class II clones grew slower and less efficiently on glucose than TMB3001 and exhibited significantly increased overflow metabolism to acetate and glycerol (Figure 4), indicating a drastic reorganization of central metabolism.

The incapability of the class I subpopulation to grow anaerobically on sole xylose is surprising because it stably propagated in the anaerobic selection chemostat. Moreover, the maximum anaerobic growth rate of all isolated clones on xylose was significantly lower than the dilution rate in the anaerobic selection chemostat, thus these clones would be expected to wash-out. The most likely explanation for this obvious discrepancy is the O\(_2\) contamination (< 200 ppm) in the technical N\(_2\) that was used to establish anaerobiosis in the bioreactor. This contamination was independently verified by mass spectroscopy (data not shown). Although incapable of anaerobic growth on xylose even in the presence of contaminating O\(_2\), we could show that the class I clone TMB3001C5 can grow on ethanol, and possibly other metabolic by-products of the class II clones, with the contaminating O\(_2\) as an external electron acceptor. Likewise, the class II clone TMB3001C1 grows significantly faster then the D of the anaerobic selection chemostat when cultivated under conditions with contaminating O\(_2\). This view is also consistent with the obvious absence of a strong selection pressure for high anaerobic growth rate on xylose during chemostat selection, since faster growing clones were readily selected within comparatively few generations in strict anaerobic batch culture.
Chapter 2

The applied strategy is a fruitful combination of rational metabolic engineering to render a strain amenable for selection and evolutionary techniques. Recently, two industrial ethanol-producing strains were metabolically engineered with the same xylose-utilization pathway that was used here (36). Compared to these industrial strains, the evolved strains shown here accumulate less xylitol and some clones have higher xylose consumption rates (e.g. TMB3001C1). Moreover, the engineered industrial strains produced only about 8% more ethanol than TMB3001 from a mixture of glucose and xylose (36), while our best clone TMB3001C5 produced about 19% more ethanol than TMB3001 (Figure 3). The presented evolutionary engineering of enabling or improving substrate utilization is not confined to the recombinant strain used here, but can in principle be applied to other substrates or organisms; e. g. the above industrial strains. As is the case for many pentoses in yeasts (6, 16), the organism subjected to selection should have the genetic potential to utilize the new substrate. Evolution may then be used to improve substrate utilization or to improve it under novel conditions. While simpler traits may be directly selected for (21, 28), more complex, multi-gene modifications require an evolution approach for step-wise improvements (25).

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The molecular basis for anaerobic growth of
Saccharomyces cerevisiae on xylose investigated by
global gene expression and metabolic flux analysis

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SUMMARY

Yeast xylose metabolism is generally considered to be restricted to respirative conditions because the two-step oxidoreductase reactions from xylose to xylulose impose an anaerobic redox imbalance. We have recently evolved, however, a *Saccharomyces cerevisiae* strain that is at present the only known yeast capable of anaerobic growth on sole xylose. Based on global gene expression and metabolic flux analysis, we identify here the distinguishing characteristics of this unique phenotype. Firstly, transcript levels and metabolic fluxes throughout central carbon metabolism were significantly higher than in the parent strain, most pronounced in the xylose-specific, the pentose phosphate, and the glycerol pathways. Secondly, differential expression of many genes involved in redox metabolism indicates that increased cytosolic NADPH formation and NADH consumption enable the higher flux through the two-step oxidoreductase reaction of xylose to xylulose in the mutant. These coordinated changes were probably brought about by a limited number of regulatory mutations. Redox balancing is apparently still a problem in this strain, since anaerobic growth on xylose could be further improved by providing an external NADH sink. This further improved growth was not accompanied by an increased xylose uptake rate nor by an increased cytosolic NADPH production rate. Because only the ATP production rate was increased in the presence of the external NADH sink, we conclude that in general anaerobic growth of yeast on xylose is primarily limited by the rate of ATP production and not by the redox balance per se.
INTRODUCTION

The yeast Saccharomyces cerevisiae serves as the paradigm model for lower and often also higher eukaryotes. In sharp contrast to almost all other organisms, however, S. cerevisiae has been domesticated for millennia and uncountable generations in human food processing. This premier status as a workhorse was earned, and continuously selected for, by the capability to anaerobically ferment sugars to ethanol and carbon dioxide at high rates. Perhaps for this reason, it is often forgotten that only some fungi (e.g. in the rumen) (38) and few yeasts grow anaerobically, and that S. cerevisiae stands out as a yeast capable of rapid anaerobic growth (44). This inability is generally related to biosynthetic hydroxylations so that oxygen can be seen as a growth factor. In fact, supplementation with sterols and unsaturated fatty acids is a prerequisite for anaerobic growth of yeasts, including S. cerevisiae, because molecular oxygen is required for the biosynthesis for membrane sterols (1). Further limitations must exist though to explain impaired anaerobic growth of yeasts excluding S. cerevisiae, even on the most common substrate glucose when supplemented with sterols. One such case is pyrimidin biosynthesis because expression of the URA1 gene from S. cerevisiae confers the ability for anaerobic growth to Pichia stipitis on glucose (36). Another, perhaps more general limitation for anaerobic growth is a sufficiently high rate of ATP formation from fermentation of a given substrate (43).

With the commercial interest in fermenting renewable resources to ethanol, significant efforts focused on metabolic engineering of efficient anaerobic pentose metabolism in S. cerevisiae (3, 11, 14, 28). A particular focus was on xylose that is not normally utilized by S. cerevisiae. Although few xylose-metabolizing yeasts are found in nature, none can grow anaerobically on xylose (15, 20). In contrast to direct isomerization of xylose to xylulose in bacteria, and possibly few fungi (13, 22), that can grow on xylose in the absence of oxygen, yeast xylose catabolism generally proceeds through the consecutive redox reactions catalyzed by NADPH-dependent xylose reductases and NAD+-dependent xylitol dehydrogenases with xylitol as the intermediate (Figure 1). By respiring NADH and supplying NADPH through the pentose phosphate pathway, which is rather active in the xylose-utilizing yeast Pichia stipitis (8), yeast metabolism can efficiently drive these coupled redox reactions for respirative growth but not for fermentative growth. Functional expression of xylose isomerase in recombinant xylose-utilizing S. cerevisiae, however, does not suffice to enable anaerobic growth on xylose (13, 21, 32, 49), which indicates that limitations beyond redox balancing must exist.
Figure 1. Bioreaction network of S. cerevisiae central carbon metabolism. Extracellular metabolites are written in capitals. ETC: Electron transport chain.
While the capability for anaerobic growth on pentoses has apparently not evolved in yeasts, we have recently isolated such a mutant from a 460 generation continuous culture (37). This evolutionary engineering experiment (33) was initiated with the xylose-utilizing \textit{S. cerevisiae} strain TMB3001 that overexpresses the NAD(P)H-dependent xylose reductase and the NAD$^+$-dependent xylitol dehydrogenase from \textit{P. stipitis} and the endogenous xylulokinase (7). Under carbon-limited selective pressure towards anaerobic growth on xylose, TMB3001 evolved into a population with improved xylose utilization (37). As a representative of the major sub-population in the evolved culture, the CI mutant was capable of anaerobic growth on sole xylose. To the best of our knowledge, \textit{S. cerevisiae} CI is at present the only yeast capable of strict anaerobic growth on xylose.

This CI strain provides a unique opportunity to elucidate the molecular mechanisms that are necessary for yeast xylose metabolism under anaerobic conditions. Using DNA microarray (6) and metabolic flux analysis (34, 41, 46), we identify here the key components that enable this phenotype. While the results reveal that balancing of redox equivalents is one crucial component, we provide strong evidence that it is ultimately the rate of ATP formation that limits anaerobic growth on xylose.
MATERIALS AND METHODS

Strains, media, and cultivation conditions

The *S. cerevisiae* strain TMB3001 (CEN.PK 113-7A (MATa, his3-Δ1, MAL2-8c, SUC2) his3::YIpXR/XDH/XK) (7) and its evolved mutant C1 (DSM 15519) (37) were used throughout. Cultures were stored in aliquots supplemented with 15% glycerol at -80°C and were revived by growth in YPD medium (10 g l\(^{-1}\) yeast extract, 20 g l\(^{-1}\) peptone, and 20 g l\(^{-1}\) glucose). All cultures for physiological and DNA microarray experiments were grown in minimal medium (37, 42). For anaerobic cultivation, the medium was supplemented with ethanol-dissolved ergosterol (Fluka) and Tween 80 (Sigma) at final concentrations of 0.01 g l\(^{-1}\) and 0.42 g l\(^{-1}\), respectively.

Flask cultures were grown in 500 ml baffled shake flasks with 50 ml medium at 30°C and 300 rpm. Carbon-limited chemostat cultures were grown in 1 l medium in a 2 l stirred tank reactor (Bioengineering, Wald, Switzerland) at a dilution (growth) rate of 0.05 h\(^{-1}\). The volume was kept constant by continuous removal of excess culture broth through a sterile needle that was fixed at a predetermined height. A constant pH of 5.0 was maintained by automatic addition of 2 M KOH. Sparging with air at a rate of 1 l min\(^{-1}\) established aerobic conditions, whereas sparging with pure N\(_2\) (O\(_2\) < 5 ppm) at a rate of 0.35 l min\(^{-1}\) established anaerobiosis. Constant gas flow rates were controlled by a mass flowmeter (Inceltech, Toulouse, France). To ensure anaerobiosis, the feed medium was also maintained under a N\(_2\) atmosphere. The stirrer speed was set to 1,000 or 500 rpm under aerobic or anaerobic conditions, respectively. Culture aliquots for metabolic flux and transcript analysis were withdrawn in physiological steady state, defined as stable cell density and rate of CO\(_2\) evolution for at least three volume changes.

Anaerobic batch experiments for metabolic flux analysis were done in Hungate tubes (Bellco Glass Inc., Vineland, NJ) that were sealed with butyl-rubber septa, using inocula that were washed twice with PBS (8 g l\(^{-1}\) NaCl, 0.2 g l\(^{-1}\) KCl, 1.44 g l\(^{-1}\) Na\(_2\)HPO\(_4\), 0.24 g l\(^{-1}\) KH\(_2\)PO\(_4\), pH 7.0). The basic salt solution was sparged with pure N\(_2\) (O\(_2\) < 5 ppm) for 15 min and, upon autoclaving, the remaining filter-sterilized, N\(_2\)-sparged medium components and 10 g l\(^{-1}\) xylose or glucose were added. Where indicated, acetoin was added at a final concentration of 0.5 g l\(^{-1}\). Withdrawal of culture aliquots was done under purging with pure N\(_2\).
RNA isolation and DNA microarray analysis

Two 50 ml culture aliquots were harvested in liquid N$_2$ pre-cooled polypropylene tubes (Greiner, Kremsmünster, Austria) and immediately centrifuged at 5,000 rpm and 4°C for 3 min. The pellets were washed twice with ice-cold AE buffer (50 mM Na-acetate, 10 mM EDTA, pH 5.2) and rapidly frozen in liquid N$_2$ for storage at -80°C. Total RNA was extracted by the hot-phenol-method (35), and the absorbance at 260 and 280 nm was used for quantification and purity control. RNA integrity was assessed in formaldehyde-containing agarose gels (31). mRNA isolation, cDNA synthesis, in vitro transcription (cRNA synthesis), and cRNA fragmentation were performed according to the Affymetrix expression analysis technical manual. Hybridization, washing, staining, and scanning of the Gene Chip Yeast Genome S98 Arrays (Affymetrix) were performed in a hybridization oven (Affymetrix), the Fluidics Station 400 (Affymetrix), and the GeneArray Scanner (Affymetrix).

Gene expression data were analyzed using the Microarray Suite 5.0 software (Affymetrix). The average fluorescence of each array was normalized to a common value of 100. From the 9,335 transcripts present on the YG-S98 array, only the 6,383 yeast open reading frames were considered (4). The coefficient of variation (CV = standard deviation divided by the mean) was calculated for all 6,383 transcripts from duplicate experiments, and the average coefficient of variation over the entire array was used to assess the experimental error of gene expression analysis in each experiment. Since low transcript levels are inherently difficult to quantify, all expression values below 20 were set to a value of 20 for fold-change analysis. Statistical analysis of differential gene expression was performed with the Significance Analysis of Micro-arrays (SAM version 1.21) EXCEL add-in software (39), because it scales down better to small numbers of replicates than the standard t-test (25). Since a fold-change of at least two with an expected median false positive rate of 1% determined by SAM were the minimum requirements for interlaboratory microarray reproducibility (4, 25), we chose these cut-off parameters for a first genome-wide gene expression analysis. To identify significant expression changes below the two-fold cut-off in genes of central carbon metabolism, we used SAM with a median false discovery rate of 1%, but without a fold-change cut-off.

Analytical methods

Cell growth was monitored by following the optical density at 600 nm (OD$_{600}$). Cellular dry weight (DW) was determined from at least five 10 ml culture aliquots that were
centrifuged at 5,000 rpm for 20 min in pre-weighed glass tubes, washed once with water, and
dried at 110°C for 24 h to a constant weight. Commercially available kits were used for enzymatic
determination of glucose (Beckman), xylose (Medichem, Steinenbronn, Germany),
xyitol (R-Biopharm, Darmstadt, Germany), acetate (R-Biopharm), and glycerol (Sigma). Ethanol, acetoin, and butanediol concentrations were determined by GC as described before (34). Pyruvate and succinate concentrations were determined by HPLC (Perkin Elmer, Shelton, Connecticut), with a Supelco H column (Supelco, Bellefonte, PA) and 0.15% H3PO4 as the mobile phase. CO2 and ethanol concentrations in the reactor off-gas were determined with a Prima 600 mass spectrometer (Fisons Instruments, Uxbridge, England).

**Determination of physiological parameters and intracellular metabolic fluxes**

Maximum exponential growth rates in batch culture were determined by log-linear regression of OD600 versus time with growth rate (μ) as the regression coefficient. The specific biomass yield (Yx/s) was determined as the coefficient of linear regression of biomass concentration versus substrate concentration (S) during the exponential growth phase. The biomass concentration was estimated from predetermined OD600-to-DW correlations during the mid-exponential growth phase of aerobic cultures on glucose for TMB3001 and C1 (0.530 and 0.479 g OD600 g"1, respectively). During the exponential growth phase, specific glucose or xylose uptake rates were calculated as the ratio between μ and Yx/s. Ethanol, xylitol, acetate, glycerol, and butanediol yields were calculated by linear regression of by-product concentration versus substrate concentration, their specific production rates were calculated as the product of specific xylose or glucose uptake rate and the by-product yield.

In chemostat cultures, biomass and by-product yields were determined as the ratio of the molar carbon in the considered product and the total molar carbon in the consumed substrates in steady state, assuming a ratio of 0.476 g(C) g(biomass)"1 (46). Specific consumption and production rates were calculated as the ratio of the considered molar production rates and the steady state biomass concentration. The fraction of evaporated ethanol, O2, and CO2 in the bioreactor off-gas were determined by on-line MS analysis.

A previously developed stoichiometric model (46) was used to estimate intracellular carbon fluxes in anaerobic chemostat and batch cultures. The fluxes to ethanol and CO2 were defined as free fluxes, whose computed values were compared with the redundant experimental ethanol and CO2 production rates. The computed free fluxes were always within 15% of the experimental values, thus confirming the reliability of the stoichiometric model.
used. This model was extended with acetoin reduction to butanediol (47) for cases where acetoin was added. The macromolecular cell composition was assumed to be 39% (w/w) polysaccharides, 50% (w/w) protein, and 6% (w/w) RNA in chemostat culture, and 40% (w/w) polysaccharides, 52% (w/w) protein, and 3% (w/w) RNA as well as 31% (w/w) polysaccharides, 56% (w/w) protein, and 9% (w/w) RNA in batch cultures on xylose and glucose, respectively (46). Since the recombinant xylose reductase in our strains can also catalyze the NADPH-dependent reduction of dihydroxyacetone-P (17), we assessed the effect of changing the co-substrate specificity of the dihydroxyacetone-P dehydrogenase from NADH to NADPH. The effect was strictly local with a higher fraction of NADH-dependent xylose reduction (data not shown). Since we do not know the in vivo concentrations of NADH and NADPH, we do not know the in vivo co-factor preference of xylose reductase (7). Hence, we were not able to determine the proportion between the NADH- and the NADPH-dependent xylose reduction fluxes.

**Enzymatic assays**

Cell extracts were prepared from mid-exponential growth phase cultures in minimal medium with glucose. Cell pellets were harvested by centrifugation, washed with deionized water, and resuspended in a 0.1 M triethanolamine buffer (pH 7.0), containing 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithio-threitol, and 0.5 mM EDTA. The suspension was vortexted with glass beads (0.5 mm diameter) at 4°C for 5 min, incubated on ice for 5 min, and vortexted again for 5 min. Cell debris and glass beads were separated by centrifugation at 20,000 x g and 4°C for 5 min. In vitro activities of xylose reductase (with NADPH and NADH), xylitol dehydrogenase (with NADH), and xylulokinase were determined in the supernatant using a previously described method (7), which was adapted for measurement in 96-well plates in a total volume of 200 μl. The sole difference was the use of triethanolamine buffer at pH 7.0 instead of glycine buffer at pH 9.0 for the xylitol dehydrogenase assay. The total protein content of the supernatant was determined with a commercially available kit (Beckman). Specific activities were expressed as units per mg of protein, where one unit is defined as reduction or oxidation of one micromole of NAD(P)H per min.
RESULTS

Chemostat cultivation

For reliable and meaningful identification of specific differences between mutants, in particular when using genome-wide methodologies, it is important to ensure identical environmental conditions and to minimize physiological differences that cause unspecific responses (6, 25). To avoid unspecific, growth rate-related effects, we grew *S. cerevisiae* TMB3001 (7) and the evolved C1 strain (37) in carbon-limited chemostat cultures at an identical growth rate for genome-wide transcript level and metabolic flux analysis. The dilution rate of 0.05 h\(^{-1}\) was chosen as the highest value that allowed reliable establishment of a physiological steady state for both strains under all conditions used. Anaerobic growth on sole xylose was not investigated directly because the control strain could not grow at all under these conditions and C1 can grow only at a maximum growth rate of 0.012 h\(^{-1}\) (37). In steady state, the C1 strain consumed xylose at a higher specific rate and left significantly lower residual xylose concentrations in the medium (Table 1). This higher co-utilization of xylose enabled much higher accumulation of biomass in the aerobic C1 culture. Under anaerobic conditions C1 and TMB3001 attained comparable biomass concentrations but the C1 strain generated significantly more metabolic by-products from the additionally consumed xylose, in particular glycerol and xylitol. In contrast to C1, TMB3001 was incapable to propagate aerobically on 20 g/L xylose as the sole carbon source (Table 1).

Global gene expression analysis of chemostat culture cells

DNA microarray analysis was done with RNA isolated from cells in the above chemostat cultures to elucidate molecular changes that underlie the capability to grow on xylose as the sole carbon source. For each strain and condition, transcript levels were quantified from duplicate experiments with average coefficients of variation between 0.13 and 0.34 (Table 2). In total, 577 genes exhibited statistically significant greater or equal than two-fold differential expression pattern (using SAM version 1.21 with a maximal expected median false positive rate of 1%) (4, 25) in C1 when compared to TMB3001 under at least one cultivation condition, 119 of which were differentially expressed under all three conditions (data not shown). For growth on sole xylose, differential gene expression was determined by comparison with TMB3001 under the same condition but with the additional carbon source glucose, since TMB3001 cannot grow at this rate on xylose (12, 37).
TABLE 1. Metabolite concentrations and physiological parameters \* of *S. cerevisiae* TMB3001 and C1 in steady state of chemostat cultures at a dilution rate of 0.05 h\(^{-1}\).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Feed concentration (g l(^{-1}))</th>
<th>Steady state concentrations * (g l(^{-1}))</th>
<th>Specific consumption or production rates (g g(^{-1}) biomass)(^{-1}) h(^{-1})</th>
<th>Carbon balance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>Xylose</td>
<td>Glucose</td>
<td>Xylose</td>
</tr>
<tr>
<td>Aerobic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMB3001</td>
<td>9.6</td>
<td>9.7</td>
<td>0</td>
<td>5.5</td>
</tr>
<tr>
<td>C1</td>
<td>9.6</td>
<td>9.7</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>C1</td>
<td>0</td>
<td>20.9</td>
<td>0</td>
<td>0.8</td>
</tr>
<tr>
<td>Anaerobic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMB3001</td>
<td>10</td>
<td>10.2</td>
<td>0.1</td>
<td>7.4</td>
</tr>
<tr>
<td>C1</td>
<td>10</td>
<td>10.2</td>
<td>0.1</td>
<td>4.7</td>
</tr>
</tbody>
</table>

\*Acetate was below detection level of 0.05 g l\(^{-1}\) with exception of the anaerobic C1 culture with 0.1 g l\(^{-1}\).

\*Mean values from two independent cultures. Standard deviations were always below 10% of the mean.

\*Values include ethanol evaporation.

A significant portion of the differentially expressed genes encoded for metabolic functions, and the by far strongest up-regulation was evident for the galactose metabolism genes *GAL1*, *GAL2*, *GAL7*, and *GAL10* under all conditions (Table 3). Expression of the central carbon metabolism genes *SOL3*, *MAE1*, *GPD2*, *ADH5*, and in particular *PYK2* was generally significantly increased, whereas *PDC6*, *ADH4*, *PGM2*, *SDH1*, *ZWF1*, *YAT1*, *ACH1* and *RHR2* were significantly over-expressed under at least one condition (Table 3). Although reported to transport also xylose (12), the hexose transporter *HXT2* was down-regulated under all conditions and *HXT4*, *HXT5*, and *STL1* under aerobic conditions (data not shown). The *HXT16* gene, in contrast, was strongly up-regulated under aerobic conditions (Table 3). Generally, we noted lower aerobic expression of several genes encoding minor or putative isoenzymes in central carbon metabolism (*TKL2*, *SOL4*, *YGR043C*, and *ALD3*), and lower anaerobic expression of the peroxysomal genes *ICL1* and *IDP3*, as well as the glycerol kinase *GUT1* and the mitochondrial glycerol-3-P dehydrogenase *GUT2* genes in C1 (data not shown). This down-regulation of genes that are responsible for glycerol consumption matches the higher expression of the glycerol producing genes *GPD2* and *RHR2* and the significantly increased glycerol production in the anaerobic C1 culture (Table 1). Furthermore, increased expression of the high capacity and low affinity ammonium transporter *MEP3*, and concomitantly decreased expression of the low capacity and high affinity ammonium transporter *MEP2*, demonstrate that C1 had adapted to the nitrogen-excess and carbon-limited...
conditions in the evolution chemostat (37). Among the most consistently and strongly down-regulated genes were the mating type-specific genes *MFA1* and *STE2*. Since decreased expression of mating type-specific genes is characteristic for diploid or polyploid strains (10), we verified haploidy and mating type of the C1 strain by PCR (data not shown).

### TABLE 2. Average coefficients of variation for the microarray experiments.

<table>
<thead>
<tr>
<th>Cultivation conditions</th>
<th>Strain</th>
<th>Average coefficient of variation&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aerobic</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>glucose/xylose</td>
<td>TMB3001</td>
<td>0.16</td>
</tr>
<tr>
<td>glucose/xylose</td>
<td>C1</td>
<td>0.13</td>
</tr>
<tr>
<td>Xylose</td>
<td>C1</td>
<td>0.34</td>
</tr>
<tr>
<td><strong>Anaerobic</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>glucose/xylose</td>
<td>TMB3001</td>
<td>0.17</td>
</tr>
<tr>
<td>glucose/xylose</td>
<td>C1</td>
<td>0.17</td>
</tr>
</tbody>
</table>

<sup>a</sup> Is the average of the coefficients of variation (deviation divided by the average from two independent cultures) for all genes.

Upon detailed inspection of metabolic functions specified by differentially expressed genes, we noted a general pattern: central carbon metabolism genes directly involved in cytosolic NADPH formation (*ZWF1*) or NADH consumption (*ADH4, ADH5, and GPD2*) were up-regulated and those involved in NADH formation (*ALD3* and *GUT2*) were down-regulated. Consistently, increased aerobic expression of the respiratory genes *COR1* and *CYT1* indicates increased NADH oxidation in C1. In addition, we observed higher expression levels of the NADPH-producing mitochondrial malic enzyme, which has been previously hypothesized to constitute a transhydrogenation system that re-oxidizes cytosolic NADH by reduction of mitochondrial NADP<sup>+</sup> (2). Similarly, up-regulation of the NADPH-dependent glutamate dehydrogenase *GDH3* indicates operation of a transhydrogenation cycle catalyzed by *GDH1, GDH2* and *GDH3* (5), which may re-oxidize NADH by reducing NADP<sup>+</sup>. The putative transhydrogenation mechanisms would be, however, irrelevant under fermentative conditions because of the very low tricarboxylic acid cycle flux (9, 23, 24).
**TABLE 3.** Metabolism-related genes with significantly increased expression levels in CI relative to TMB3001 under at least one chemostat condition.

<table>
<thead>
<tr>
<th>Name</th>
<th>ID</th>
<th>Fold-change of gene expression in chemostat culture</th>
<th>Gene description</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Aerobic Glucose Xylose</td>
<td>Anaerobic Glucose Xylose</td>
</tr>
</tbody>
</table>
Comparison between transcript level and intracellular carbon fluxes

The large number of differentially expressed genes in central carbon and redox metabolism suggested significant metabolic differences between TMB3001 and Cl. Hence we estimated the intracellular flux distribution from the anaerobic chemostat data of both strains on the glucose/xylose mixture (Table 1) with a previously described stoichiometric model (46). Enhanced xylose catabolism in the Cl culture resulted in about 20% increased fluxes throughout the entire network but more than doubled fluxes through the pentose phosphate pathway and into glycerol formation (Figure 2). Notably, the flux of glucose-6-P into the pentose phosphate pathway was strongly increased in Cl, which is consistent with the notion that more cytosolic NADPH is required in the mutant to drive xylose reduction.

Generally, increased carbon fluxes in the Cl strain were in excellent agreement with increased transcript levels of the corresponding genes from the same chemostat culture (Figure 2). Albeit in many cases below the two-fold significance level, it is remarkable that a statistically significant and consistent increase in absolute and expression levels was discernible for most relevant central metabolic genes. This expression increase was of about the same scale as the flux increase; i.e. SOL1, SOL3, GND1, XKS1, TKL1, and TAL1 in the pentose phosphate pathway, PFK1, PFK2, TDH1, GPM1, GPM2, GPM3, ENO2, PYK1, and PYK2 in glycolysis, and GPD1, GPD2, RHR2, PDC1, ADH1, ADH3, and ADH5 in the by-product pathways. Among the generally overexpressed ADH genes, ADH2 was clearly unchanged. This is fully consistent with the function of ADH2 in metabolizing ethanol (27, 40) that is not required for anaerobic ethanol formation. Among the expression levels of the ALD genes involved in acetate formation, in contrast, only ALD5 expression was increased significantly, as would be expected from the very low flux increase through this pathway.

Since the recombinant xylose reductase and xylitol dehydrogenase were not present on the YG-S98 array, we determined the in vitro enzymatic activities of the xylose catabolism enzymes from batch cultures of TMB3001 and Cl (Table 4). The Cl strain exhibited drastically higher activities of all three enzymes, in particular a five-fold higher xylose reductase activity. Indeed, as observed in Cl, increased overexpression of the xylose reductase has been previously reported to increase xylose uptake rate, glycerol and acetate yields, as well as decrease xylitol yield (17). The redox cofactor specificity of xylose reductase remained unaltered at an about 100% higher activity with NADPH. While uncontrolled xylulokinase over-expression is detrimental to S. cerevisiae (18, 19), more than
doubled activity is without any apparent negative effect in Cl, presumably because evolution increased all three enzyme activities simultaneously.

Figure 2. Comparison of molar carbon fluxes (ovals; mMol g(biomass)⁻¹ h⁻¹) and transcript levels of the related enzymes (boxes; arbitrary units) in S. cerevisiae TMB3001 (upper values) and Cl (lower values) during anaerobic chemostat cultivation on 10 g l⁻¹ each of glucose and xylose. The average and the deviation of DNA microarray analysis from two independent cultures are shown. Independent of the fold-change, changes in gene expression were considered as significant (highlighted in bold numbers) upon SAM analysis with an expected median false positive rate of 1%. The deviation of all carbon fluxes was below 10% of the average from two independent cultures. Extracellular metabolites are written in capitals. Fluxes from the following intracellular metabolites are required for biomass formation but are not shown explicitly: glucose-6-P, ribose-5-P, erythrose-4-P, 3-phosphoglycerate, phosphoenolpyruvate, pyruvate, acetate (24, 46).
TABLE 4. Specific enzyme activities (U mg(protein)^{-1}) in the catabolic xylose pathway of *S. cerevisiae* TMB3001, and C1 during aerobic batch growth on glucose.

<table>
<thead>
<tr>
<th></th>
<th>xylose reductase</th>
<th>xylitol dehydrogenase</th>
<th>xylulokinase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NADPH</td>
<td>NADH</td>
<td></td>
</tr>
<tr>
<td>TMB3001</td>
<td>0.08 ± 0.00^a</td>
<td>0.04 ± 0.00</td>
<td>0.8 ± 0.0</td>
</tr>
<tr>
<td>C1</td>
<td>0.42 ± 0.03</td>
<td>0.24 ± 0.01</td>
<td>1.9 ± 0.0</td>
</tr>
</tbody>
</table>

^aAverage value and deviation from two independent experiments.

The large number of differentially expressed genes in central carbon metabolism and their consistent expression level suggests few regulatory rather than many specific mutations in the C1 strain. Supporting this view, we found statistically significant increased expression of the positive regulators of glycolysis and ethanol formation *GCR1, REB1, RAP1* and *PDC2* (Table 5). The extraordinary up-regulation of *GAL3* appears to override the regulation exerted by the moderately increased expression of the galactose repressors *GAL80* and *GAL6* (Table 5), thus being responsible for the strong induction of the *GAL* genes (Table 3). Increased expression of many components from the high osmolarity glycerol (HOG) regulatory pathway (50) was probably responsible, at least in part, for the up-regulation of genes involved in glycerol formation. Lastly, increased expression of the negative regulator of hexose transport, *MTH1*, may be the common cause for the down-regulation of *HXT2, HXT5, HXT4*, and *STL1*.

**Increased catabolic rates improve anaerobic growth on xylose**

Directed evolution of the C1 strain had apparently relieved the metabolic limitations for anaerobic growth on xylose (37), and the above identified molecular differences in C1 suggest that the coordinated changes in carbon and redox metabolism are key components of this phenotype. These analyses reveal not, however, the pivotal limitation of yeast pentose metabolism for anaerobic growth, but the evolved strain provides a unique opportunity to address this question experimentally. Generally, the stoichiometry of anaerobic glucose metabolism was not radically different from that of anaerobic xylose metabolism, although less biomass was generated per gram of consumed substrate in the latter case (Table 6). The maximum specific rate of growth, in contrast, was dramatically lower on xylose (Table 6). This indicates a kinetic problem, which may reside in uptake and/or catabolism of xylose or in
an insufficient rate of ATP production to support growth, as was hypothesized previously (29).

TABLE 5. Regulatory genes of sugar transport, osmotic stress response, central carbon and galactose metabolism with significantly changed expression levels in C1 relative to TMB3001 under at least one chemostat condition.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold-change of gene expression in chemostat culture</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Aerobic</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>Name</td>
<td>ID</td>
<td>Xylose</td>
<td>Glucose</td>
</tr>
<tr>
<td>Sugar transport</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>xylose</td>
<td>MTH1 YDR277C</td>
<td>2.1</td>
<td>2.6</td>
</tr>
<tr>
<td>Central carbon metabolism</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADR1 YDR216W</td>
<td>1.0</td>
<td>0.8</td>
<td>0.1</td>
</tr>
<tr>
<td>CYC8 YBR112C</td>
<td>2.2</td>
<td>2.0</td>
<td>1.5</td>
</tr>
<tr>
<td>GCR1 YPL075W</td>
<td>1.9</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>HAP1 YLR256W</td>
<td>1.7</td>
<td>2.0</td>
<td>1.4</td>
</tr>
<tr>
<td>HAP5 YOR358W</td>
<td>2.5</td>
<td>2.8</td>
<td>2.1</td>
</tr>
<tr>
<td>PDC2 YDR081C</td>
<td>1.9</td>
<td>1.7</td>
<td>1.2</td>
</tr>
<tr>
<td>RAP1 YNL216W</td>
<td>2.0</td>
<td>1.9</td>
<td>1.0</td>
</tr>
<tr>
<td>REB1 YBR049C</td>
<td>1.6</td>
<td>2.1</td>
<td>0.8</td>
</tr>
<tr>
<td>SNF1 YDR477W</td>
<td>2.0</td>
<td>1.8</td>
<td>1.0</td>
</tr>
<tr>
<td>TUP1 YCR084C</td>
<td>1.8</td>
<td>2.6</td>
<td>1.0</td>
</tr>
<tr>
<td>Galactose metabolism</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAL3 YDR009W</td>
<td>14.6</td>
<td>21.1</td>
<td>7.5</td>
</tr>
<tr>
<td>GAL4 YPL248C</td>
<td>2.1</td>
<td>2.1</td>
<td>0.4</td>
</tr>
<tr>
<td>GAL6 YNL239W</td>
<td>2.7</td>
<td>2.6</td>
<td>2.7</td>
</tr>
<tr>
<td>GAL80 YML051W</td>
<td>3.5</td>
<td>4.1</td>
<td>2.1</td>
</tr>
<tr>
<td>Stress responses (HOG-pathway,..)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASK10 YGR097W</td>
<td>2.2</td>
<td>1.7</td>
<td>2.5</td>
</tr>
<tr>
<td>MSN1 YOL116W</td>
<td>2.6</td>
<td>2.3</td>
<td>1.3</td>
</tr>
<tr>
<td>MSN4 YKL062W</td>
<td>2.6</td>
<td>2.4</td>
<td>2.1</td>
</tr>
<tr>
<td>PBS2 YJL128C</td>
<td>1.4</td>
<td>1.3</td>
<td>2.9</td>
</tr>
<tr>
<td>PTP2 YOR208W</td>
<td>2.9</td>
<td>2.0</td>
<td>2.1</td>
</tr>
<tr>
<td>RIM15 YFL033C</td>
<td>2.2</td>
<td>1.7</td>
<td>1.1</td>
</tr>
<tr>
<td>SKN7 YHR206W</td>
<td>1.8</td>
<td>2.0</td>
<td>0.8</td>
</tr>
<tr>
<td>SSK22 YCR073C</td>
<td>2.9</td>
<td>2.1</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Gene expression levels were considered significant upon at least two-fold SAM-determined changes at an expected median false positive rate of 1%.

Compared to TMB3001 cultivated under aerobic conditions on xylose and glucose.

Expression change does not meet the two-fold cut-off, but was statistically significant with an expected median false positive rate of 1%.

To differentiate between these possibilities, we grew C1 anaerobically on xylose in the presence of acetoin, which can be reduced to butanediol by consuming NADH. This reaction reduces extensive cytosolic NADH formation in the xylitol dehydrogenase reaction (Figure 1), and thus increases the rate of xylose catabolism during anaerobic xylose fermentation (47).
Acetoine was quantitatively converted to butanediol in the C1 culture (data not shown) and less xylitol accumulated in the presence of acetoine, presumably as a consequence of NADH oxidation (Table 6). While the specific rate of xylose uptake remained virtually identical, the rate of xylose catabolism increased significantly because ethanol was produced instead of xylitol. Notably, co-metabolism of acetoine increased the rate of anaerobic growth and the biomass yield on sole xylose by about one third (Table 6), thus clearly demonstrating that xylose uptake is not the limiting factor for anaerobic growth of yeast on sole xylose.

**TABLE 6. Physiological parameters of TMB3001 on glucose and of C1 on xylose in anaerobic batch cultures with 10 g l\(^{-1}\) carbon source (C-source).**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Substrate</th>
<th>Maximum growth rate (^a) ((h^{-1}))</th>
<th>Specific substrate uptake rate (^b) ((g g(\text{biomass})^{-1}h^{-1}))</th>
<th>Yields of (^c)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(gX g(^{-1}))</td>
<td>(g g(^{-1}))</td>
<td>(g g(^{-1}))</td>
<td>(g g(^{-1}))</td>
</tr>
<tr>
<td>TMB3001</td>
<td>Glucose</td>
<td>0.373</td>
<td>4.51</td>
<td>0.083</td>
<td>0.397</td>
</tr>
<tr>
<td>C1</td>
<td>Xylose</td>
<td>0.014</td>
<td>0.60</td>
<td>0.024</td>
<td>0.277</td>
</tr>
<tr>
<td>C1 + acetoin(^f)</td>
<td>xylose</td>
<td>0.019</td>
<td>0.62</td>
<td>0.031</td>
<td>0.373</td>
</tr>
</tbody>
</table>

\(^a\)Average values. The deviation was below 10%.

\(^b\)Average values. The deviation was below 5%.

\(^f\)0.5 g l\(^{-1}\) acetoin was added.

Metabolic flux analysis was then used to distinguish whether increased xylose catabolism per se or increased rate of ATP formation caused the more rapid anaerobic growth of C1 on xylose plus acetoine. As expected (23, 24), less than 4% of the consumed glucose enters the pentose phosphate pathway in the anaerobic TMB3001 culture, primarily to supply pathway intermediates for biomass formation (Figure 3A). In the C1 culture, in contrast, about 20% of the consumed xylose re-enters the oxidative pentose phosphate pathway to supply NADPH for the xylose reductase reaction (Figure 3B; upper values). From the estimated intracellular fluxes through the known ATP-consuming and -producing biochemical reactions catalyzed by xylulokinase, hexokinase, phosphofructokinase, phosphoglycerate kinase, and pyruvate kinase, we then calculated the specific ATP production rates of the three cultures. The thus calculated ATP production rate is the maximum that is available for biomass formation. The
production rate of 3.7 mMol(ATP) g(biomass)^{-1} h^{-1} of C1 on xylose was extraordinarily low, when compared to the 38.7 mMol g(biomass)^{-1} h^{-1} produced by TMB3001 during anaerobic growth on glucose. The latter rate resulted in a $Y_{ATP}$ of about 10 g(biomass) (moles ATP)^{-1} that was very similar to the previously determined value of 11 g(biomass) (moles ATP)^{-1} determined from a glucose-limited anaerobic chemostat culture (43). Finally, in the presence of acetoin, the production rate increased to 4.9 mMol g(biomass)^{-1} h^{-1} during anaerobic growth on xylose, and this 32% increase correlates almost perfectly with the 35% increase in growth rate in the presence of acetoin.

Figure 3. Molar carbon fluxes in mMol g(biomass)^{-1} h^{-1} during anaerobic exponential batch growth of TMB3001 on 10 g l^{-1} glucose (A) and C1 on 10 g l^{-1} xylose (B; upper values) or 10 g l^{-1} xylose plus 0.5 g l^{-1} acetoin (B; lower values). Averages of duplicate experiments are shown and the deviation was below 10% in all cases.
DISCUSSION

Based on global gene expression and metabolic flux analysis, we identified two distinguishing characteristics that are seemingly necessary for anaerobic growth of *S. cerevisiae* on sole xylose. Firstly we showed that enhanced xylose consumption was accompanied by moderately increased carbon fluxes throughout the entire metabolic network of C1, most pronounced in the pentose phosphate and glycerol pathways. The calculated changes in flux between intracellular metabolites were in excellent agreement with the 1.5 to 2-fold higher expression level of most central metabolic genes in C1 compared to its parent TMB3001. Secondly, the pattern of differentially expressed metabolic genes suggested that increased cytosolic NADPH formation and increased cytosolic NADH consumption are important to drive xylose catabolism under anaerobic conditions. From these results we conclude that organisms such as yeasts that rely on two consecutive redox reactions for pentose catabolism are only capable of anaerobic growth on this compound when redox metabolism enables sufficiently high catabolic fluxes. Since we could not detect any mutations in the open reading frames and promoter regions of the most prominently up-regulated genes *GAL2, PYK2*, and those encoding for xylose reductase, xylitol dehydrogenase, and xylulokinase (data not shown), it appears that the altered expression of key regulator proteins is predominantly responsible for this phenotype.

Originating from a polyploid parent strain, the randomly generated *S. cerevisiae* mutant TMB3400 with improved co-utilization of xylose (48) was recently subjected to global expression analysis under almost identical conditions to those used here (45). Consistent with C1, several pentose phosphate pathway and galactose metabolism genes were up-regulated. Since Gal2p can also transport xylose (12), the consistent observation that *GAL2* was among the most strongly up-regulated genes in C1 and in TMB3400 strongly suggests that xylose uptake is at least in part mediated by Gal2p. The strong up-regulation of galactose metabolism-related genes was probably induced by the higher expression level of the dominant positive regulator *GAL3* (Table 5) (45), in particular since we were unable to detect any mutation in the upstream region of *GAL2* in our mutants. In contrast to C1, expression levels of genes involved in glycolysis, redox metabolism, and ethanol or glycerol production were not altered in TMB3400, which may explain why this mutant cannot grow anaerobically on sole xylose (48).

Our conclusion that increased cytosolic NADPH formation and NADH consumption are important for anaerobic growth of yeast on xylose concurs with flux and proteome data from
recombinant, xylose-fermenting *S. cerevisiae* during a shift from glucose to glucose/xylose containing media (26, 30). In particular, it was shown that strains overexpressing the preferentially NADPH-dependent xylose reductase from *P. stipitis* generated the required NADPH primarily through the oxidative pentose phosphate pathway (16). This causes lower glycolytic fluxes and the concomitantly reduced NADH re-oxidation by reactions downstream of fructose-6-P can be compensated in respiring but not in fermenting yeast (2); hence explaining the capacity of such recombinant strains to grow aerobically but not anaerobically on sole xylose (7, 14).

While directed evolution of C1 had apparently relieved all metabolic bottlenecks for anaerobic growth on xylose (37), the slow rate of growth with a doubling time of about 50 h shows that the biosynthetic components are barely generated at an appropriate rate. Adding the NADH-oxidizing compound acetoin to C1 cultures increased the anaerobic growth rate on sole xylose by about one third, which demonstrates that balancing of redox co-factors was still growth-limiting. Xylose uptake was clearly not limiting, since the rate of xylose uptake remained constant and xylose catabolism increased only because less of the side-product xylitol was produced. More detailed flux analysis then revealed that the specific cytosolic NADPH production rate remained constant but that the ATP production rate increased by the same factor as the growth rate in the acetoin co-feed culture. This result strongly suggests that the rate of ATP formation is the primary limiting factor for anaerobic growth on sole xylose, although we cannot exclude that the pentose phosphate pathway operates at its maximum, and thus cannot supply more NADPH.

This raises the general question, what rate of ATP formation is actually necessary to support anaerobic growth on sole xylose? While the specific ATP production rate of 3.7 mMol g(biomass)$^{-1}$ h$^{-1}$ was sufficient in C1, a rate of 1.8 mMol ATP g(biomass)$^{-1}$ h$^{-1}$ in TMB3001 upon supplementation with acetoin was apparently insufficient (47). Since the amount of ATP that is necessary to just sustain viability of existing cell mass, the so-called maintenance energy, is about 1.2 mMol ATP g(biomass)$^{-1}$ h$^{-1}$ in oxygen-limited *P. stipitis* cultures on sole xylose (29), it appears that at least 2 mMol ATP g(biomass)$^{-1}$ h$^{-1}$ must be generated from the catabolism of xylose to enable anaerobic growth. Within 460 generations under selective pressure for improved xylose metabolism (37), the C1 strain has apparently evolved such that altered redox cofactor metabolism in a number of reactions and higher expression levels of almost all catabolic genes permit sufficiently high catabolic fluxes of xylose to ethanol.
Chapter 3

Acknowledgments

We thank Jörg Hauf for mating type determination of Cl. This work was funded by the Swiss Bundesamt für Bildung und Wissenschaft within European Commission Framework V project BIO-HUG, and the Swedish Energy Administration.
REFERENCES

Chapter 3


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Investigating three potential bottlenecks of yeast xylose catabolism by rational and evolutionary engineering

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Institute of Biotechnology, ETH Zürich, CH-8093 Zürich, Switzerland

SUMMARY

This chapter attempts to address three potential problems of xylose catabolism: pentose phosphate pathway capacity, redox co-factor balancing, and xylose uptake. For this purpose, genetically engineered strains were constructed and subjected to long-term evolution experiments. We describe here the overexpression of UdhA, the soluble transhydrogenase from Escherichia coli, in Saccharomyces cerevisiae. UdhA expression restored growth on sole glucose minimal medium of a phosphoglucone isomerase deletion mutant, demonstrating that this heterologous enzyme is functional in yeast and that the pentose phosphate pathway can support growth on glucose as the only catabolic pathway in S. cerevisiae. Moreover, we found that the corresponding pentose phosphate capacity is more than twice as high as the flux determined for the xylose catabolizing recombinant TMB3001 strain during fermentation of xylose only. This indicates that the pentose phosphate flux does not limit xylose catabolism in TMB3001, at least at the current level. Overexpression of UdhA in the xylose fermenting strain TMB3001 and in its glucose-6-P dehydrogenase mutant TMB3255 did not circumvent the problem of redox balancing, probably because unfavorable thermodynamic conditions favor the undesired reaction toward NADH production and NADPH re-oxidation. Finally, even after 110 generations of selection under the successful conditions described in chapter 1, we were not able to isolate improved mutant forms of the hexose transporter HXT4 from a TMB3001 derivative deleted for all 18 hexose transporters but expressing HXT4.
Chapter 4

INTRODUCTION

Efficient fermentation of pentoses, in particular xylose, is of primary importance for the economically viable bioconversion of hemicellulose rich and cheap lignocellulosic material to ethanol (33). Due to its well known high resistance to harsh process conditions and elevated ethanol productivities on hexoses, the non-pentose fermenting yeast *Saccharomyces cerevisiae* would be an ideal protagonist for such a fermentation process (38). For that reason, a functional xylose metabolic pathway was established in backer's yeast by classical metabolic engineering (14, 17, 19). Aerobic growth and suboptimal anaerobic ethanol production on xylose were achieved by the heterologous expression of the *Pichia stipitis* NAD(P)H-depending xylose reductase and NAD\(^+\)-requiring xylitol dehydrogenase, as well as of the homologous xylulokinase (11).

This catabolic pathway, which is typical for the naturally xylose fermenting yeasts, resulted in extremely low xylose fermentation rates, as well as in xylitol accumulation under anaerobic conditions. The absence of a specific xylose transporter in *S. cerevisiae* (14) is metabolically the first problem, which this organism has to cope with for xylose catabolism. Indeed, suboptimal xylose transport occurs through different glucose transporters with higher affinity to the latter sugar (15), and inhibition of this process is observed when glucose is present in the medium. The second problem is redox balancing that becomes necessary because of different cofactor usage of xylose reductase and xylitol dehydrogenase. The third potential problem may be the limited capacity of the non oxidative pentose phosphate pathway, which is barely used in yeast growing on glucose (12, 13). These three metabolic problems are generally considered possible bottlenecks that hamper efficient xylose catabolism (14). Physiological analysis (36), metabolic engineering (20), and more recently genome-wide and whole-cell approaches (26, 28, 29, 34) demonstrated clearly that optimal anaerobic xylose catabolism is primarily compromised by the unfavorable intracellular redox status generated by this kind of catabolic pathway. Thus, engineering of redox metabolism may be the first step on the route for optimal xylose catabolism in *S. cerevisiae*.

Pyridine nucleotide transhydrogenases catalyze the transfer of reducing equivalents between the NADH/NAD\(^+\) and NADPH/NADP\(^+\) co-enzyme systems. Two classes of transhydrogenases were identified (27): AB-transhydrogenases, which are membrane bound and BB-transhydrogenases, present as soluble proteins in the cytoplasmatic fraction of many bacteria, wherein also the UdhA of *Echerichia coli* (4). Since no physiologically relevant
transhydrogenase systems exist in *S. cerevisiae* (5, 6), the introduction of this activity influenced significantly the fermentation by-product spectrum of glucose fermenting yeast (1, 24). The over-expression of a soluble transhydrogenase from *Azoto*bacter *vinelandii* in *S. cerevisiae* was reported to catalyze the production of NADH from the oxidation of NADPH, which was ascribed to the high NADPH/NADP⁺: NADH/NAD⁺ ratio present in yeast, and is not favorable for ethanol production (24, 25). Thus, the useful operation of a soluble transhydrogenase toward NADPH production and NADH re-oxidation may be exploited only when increased catabolic NADPH consumption occurs, like during xylose catabolism in yeast (20).

In this study we probe the capacity of the pentose phosphate pathway by forcing the entire glucose flux of yeast through this pathway in a phosphoglucose isomerase deletion mutant (3) expressing the soluble transhydrogenase UdhA from *E. coli* (4). Secondly, we report here the influence of the over-expression of UdhA on the xylose fermenting *S. cerevisiae* strain TMB3001 (11) with and without a functional NADPH-generating oxidative pentose phosphate pathway (20). Finally, we attempted to evolve the hexose transporter deleted TMB3201 (15), expressing only *HXT4*. The idea being that potential variants of *HXT4* with further improved xylose transport characteristics and/or possibly minimized inhibition characteristics by glucose could be identified from the plasmid-based *HXT4* expression system.
MATERIAL AND METHODS

Strains, media and culture conditions

The *S. cerevisiae* strains used throughout this study are listed in table 1. Strains were stored at -80°C in culture aliquots containing 15% glycerol. These stock cultures were used to inoculate YPD medium (10 g l-1 yeast extract, 20 g l-1 peptone, and 20 g l-1 glucose) precultures. Before starting physiological experiments, cultures were grown once in minimal medium, which was the same as described previously (29).

Aerobic batch cultures of 100 ml were grown in 1 l baffled shake flasks on a rotary shaker at 30°C and 300 rpm in minimal medium containing 5 g l-1 glucose. Uracil (50 mg l-1), tryptophan (50 mg l-1), histidine (50 mg l-1), and leucine (250 mg l-1) were supplemented where necessary. Fermentation performance on minimal medium and chemostat selections were performed as described previously (30, 31).

Screening for improved growth rates in 1 ml sole xylose minimal medium was performed in 96 deep well-plates (10), which we incubated at 30°C and 300 rpm. Pre-cultures on glucose minimal medium were washed twice with PBS (8 g l-1 NaCl, 0.2 g l-1 KCl, 1.44 g l-1 Na2HPO4, 0.24 g l-1 KH2PO4, pH 7.0) prior to inoculation of the screening cultures.

Determination of physiological parameters

In batch culture, exponential growth rates were determined by log-linear regression of OD_{600} versus time with growth rate (μ) as the regression coefficient. The specific biomass yield (Y_{DW/gl}) was determined as the coefficient of linear regression of biomass concentration (DW) versus glucose concentration during the exponential growth phase. A OD_{600}-to-DW correlation of 0.581 was assumed for ENY.WA-1A and EBY44 to estimate the biomass concentration during aerobic batch growth on glucose minimal medium. During the exponential growth phase, the specific glucose uptake rate (q_{gl}) was calculated as the ratio between μ and Y_{DW/gl}. Ethanol, xylitol, acetate and glycerol yields on glucose or xylose were calculated by linear regression of by-product concentration versus substrate concentration during exponential growth on glucose or after glucose depletion until the end of the experiment, respectively. The evaporated amount of ethanol was estimated for minimal medium fermentations by following the ethanol concentration decrease in an identical culture set-up containing 100, 50, and 25 g l^{-1} ethanol solutions instead of culture broth. An evaporation constant of 0.001 h^{-1} was determined (35).
TABLE 1. *Saccharomyces cerevisiae* strains and plasmids used throughout this study

<table>
<thead>
<tr>
<th>Strains and Plasmids</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ENY.WA-1A</td>
<td>MATa, <em>ura3</em>-52, <em>leu2</em>-3, <em>trp1</em>-289, <em>his3</em>-Δ1, <em>MAL2</em>-8c, <em>MAL3</em>, <em>SUC3</em></td>
<td>(3)</td>
</tr>
<tr>
<td>ENY.WA-1A pg1-1Δ::URA3</td>
<td></td>
<td>(3)</td>
</tr>
<tr>
<td>EBY44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMB3001</td>
<td><em>CEN.PK 113</em>-7A <em>his3</em>::Yip XR/XDH/XK</td>
<td>(11)</td>
</tr>
<tr>
<td>TMB3255</td>
<td>TMB3001 <em>zwf1</em>::KanMX</td>
<td>(20)</td>
</tr>
<tr>
<td>TMB3201</td>
<td>TMB3001 <em>Δhxt1</em>-17, <em>Δgal2</em>, <em>Δstl1</em>, <em>Δagt1</em>, <em>Δmph2</em>, <em>Δmph3</em>, <em>leu2</em>-3,112, <em>ura3</em>-52, <em>trp1</em>-289</td>
<td>(15)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P425</td>
<td>Yeast episomal vector carrying the truncated <em>HXT7</em> promoter and <em>CYC2</em> terminator</td>
<td>(16)</td>
</tr>
<tr>
<td>P426</td>
<td>Yeast episomal vector carrying the truncated <em>HXT7</em> promoter and <em>CYC2</em> terminator</td>
<td>(16)</td>
</tr>
<tr>
<td>P5UDHA</td>
<td>p425 overexpressing the <em>E. coli</em> UdhA</td>
<td>This study</td>
</tr>
<tr>
<td>P6UDHA</td>
<td>p426 overexpressing the <em>E. coli</em> UdhA</td>
<td>This study</td>
</tr>
<tr>
<td>PTHHXT4</td>
<td>p426 overexpressing the <em>S. cerevisiae</em> <em>HXT4</em></td>
<td>(15)</td>
</tr>
</tbody>
</table>

In mixed substrate fermentation analysis, the specific xylose uptake rate was determined as the ratio of the linear regression coefficient of xylose concentration versus time and the average biomass concentration between the onset of xylose consumption and the end of the fermentation experiment. In these cases, the DW was determined after glucose depletion and at the end of the experiments. The analytical methods were the same used in chapter 1.

**Generation of uracil auxotrophy**

Uracil auxotroph mutants of TMB3001 and TMB3255 were isolated by selection on 5-fluoroorotic acid (5-FOA) (Sigma) containing minimal medium agar plates (1.5 % (w/v) agar).
described like previously (2). The resulting mutants were TMB3001 (ura) and TMB3255 (ura').

**Overexpression of udhA**

The soluble transhydrogenase UdhA of *Escherichia coli* (4) was cloned as a *BamHI-HindIII* fragment from an *E.coli* overexpression vector (9) under the control of the constitutive, truncated *HXT7* promotor of the p425 (LEU2) and p426 (URA3) plasmids (16, 23) to generate p5UDHA and p6UDHA, respectively. The latter plasmids were transformed by the lithium acetate method (7) in ENY.WA-1A, EBY44, TMB3001 (ura'), and TMB3255 (ura').
RESULTS

Overexpression of UdhA in EBY44

To demonstrate in vivo functionality in yeast, we expressed the soluble transhydrogenase UdhA from E. coli in the phosphoglucone isomerase deficient S. cerevisiae strain EBY44 (pgi-1Δ). This strain is unable to grow on sole glucose, as a result of the depletion of the NADP⁺ pool caused by the channeling of the complete carbon flux through the NADPH generating oxidative pentose phosphate pathway. In a previous report, aerobic growth on glucose could be restored in a pgi-1Δ mutant by overexpressing the NADH-dependent glutamate dehydrogenase GDH2, which, in concert with the NADPH-dependent GDH3, constituted a transhydrogenase system that re-oxidizes the NADPH produced in the oxidative pentose phosphate pathway (3). Since we detected growth on sole glucose minimal medium on plate and liquid medium upon expression of UdhA in EBY44 (Table 2), we conclude that UdhA is fully functional in yeast, providing NADPH re-oxidation under aerobic conditions. Furthermore, since the maximum growth rate of EBY44-p5UDHA was still about half of the growth rate measured for its wild type strain (Table 2), it becomes evident that the pentose phosphate pathway can be rather active in S. cerevisiae. Since in a pgi-1Δ mutant the glucose uptake flux is equal to the flux through the pentose phosphate pathway, we estimated that a specific flux of about 2.94 mMol gDW⁻¹ h⁻¹ is catalyzed through this pathway.

TABLE 2. Effect of transhydrogenase over-expression on a wild type and a pgi deletion background on physiological parameters during aerobic batch growth on glucose.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth rate (h⁻¹)</th>
<th>Biomass yield (g gDW⁻¹)</th>
<th>Specific glucose uptake rate (g h⁻¹ gDW⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENY.WA-1A-p425</td>
<td>0.29 ± 0.01b</td>
<td>0.16 ± 0.01</td>
<td>1.85 ± 0.01</td>
</tr>
<tr>
<td>ENY.WA-1A-p5UDHA</td>
<td>0.31 ± 0</td>
<td>0.13 ± 0</td>
<td>2.38 ± 0.06</td>
</tr>
<tr>
<td>EBY44-p425</td>
<td>0</td>
<td>0</td>
<td>n.d.a</td>
</tr>
<tr>
<td>EBY44-p5UDHA</td>
<td>0.15 ± 0.01</td>
<td>0.27 ± 0</td>
<td>0.53 ± 0.03</td>
</tr>
</tbody>
</table>

aNot determined
bAverage values and deviations from duplicate experiments
Overexpression of UdhA in TMB3001 and TMB3255

To avoid interference with the intracellular redox metabolism of *S. cerevisiae*, an ideal xylose catabolism would be the redox-neutral isomerization of xylose to xylulose, as is achieved by bacteria (18). However, no or only insufficient xylose fermentation could be detected in strains overexpressing different xylose isomerase enzymes in *S. cerevisiae*, even after further engineering (14, 32, 37). Moreover, we were not able to achieve further improvements by subjecting the wild type CEN.PK113-6B strain (8), expressing the *Thermus thermophylus* and *Arabidopsis thaliana* xylose isomerases in combination with the homologous xylulokinase, to our previously described evolutionary engineering process (31) for more than 150 generations (data not shown). Thus, the yeasts specific oxidoreductase pathway appears to be the only effective way to catabolize xylose in *S. cerevisiae*. To improve catabolism through this pathway, however, redox balancing mechanisms are necessary (29), and transhydrogenases may provide this possibility.

To investigate the effect of transhydrogenase overexpression on redox metabolism of xylose catabolizing recombinant *S. cerevisiae*, we transformed uracil auxotroph mutants of TMB3001 with p6UDHA. Since the reduction of the NADPH-supplying oxidative pentose phosphate pathway flux was reported to decrease the intracellular NADPH concentration in the TMB3001 background (20), we analyzed UdhA overexpression also in the glucose-6-P dehydrogenase deficient strain TMB3255.

Fermentation performance analysis was performed in minimal medium batch cultures containing 50 g l⁻¹ of both glucose and xylose. In general, significant xylose fermentation was observed only after glucose depletion, when growth arrested. No significant differences during both, glucose and xylose consumption phases, could be detected when comparing TMB3001 to the transhydrogenase expressing strain TMB3001-p6UDHA (Tables 3 and 4). Like reported previously (20, 21), TMB3255 showed an about 30% lower growth rate than TMB3001 on glucose, which could be increased with methionine supplementation by about 40% (data not shown). During sole xylose consumption, a halved specific xylose uptake rate and a 50% increased ethanol yield were observed in TMB3255 compared to TMB3001. Moreover, TMB3255 exhibited double acetate yields, as well as 60% and 15% decreased xylitol and glycerol yields, respectively (Table 4). These results agree well with previously reported data (21), and no effect on xylose fermentation could be observed upon methionin addition (data not shown). Differently from TMB3001, however, we detected significant physiological changes upon UdhA expression in TMB3255 during the xylose consumption
phase. Indeed, transhydrogenase expression caused a further decrease of specific xylose uptake rate to the very low value of 0.03 g gDW\(^{-1}\) h\(^{-1}\) (Table 4).

**TABLE 3.** Physiological parameters\(^a\) during the exponential growth phase on glucose of the fermentation performance analysis performed in minimal medium with 50 g l\(^{-1}\) each of glucose and xylose.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth rate (h(^{-1}))</th>
<th>Biomass (g gDW(^{-1}))</th>
<th>Ethanol (g gDW(^{-1}))</th>
<th>Glycerol (g gDW(^{-1}))</th>
<th>Acetate (g gDW(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMB3001-p426</td>
<td>0.27 ± 0.00</td>
<td>0.06 ± 0.00</td>
<td>0.43 ± 0.00</td>
<td>0.12 ± 0.02</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>TMB3001-p6UDHA</td>
<td>0.28 ± 0.02</td>
<td>0.07 ± 0.01</td>
<td>0.46 ± 0.01</td>
<td>0.13 ± 0.03</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>TMB3255-p426</td>
<td>0.20 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>0.47 ± 0.01</td>
<td>0.11 ± 0.00</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>TMB3255-p6UDHA</td>
<td>0.19 ± 0.03</td>
<td>0.05 ± 0.01</td>
<td>0.43 ± 0.01</td>
<td>0.16 ± 0.01</td>
<td>0.05 ± 0.00</td>
</tr>
</tbody>
</table>

\(^a\)Average and deviations from duplicate experiments

**TABLE 4.** Physiological parameters\(^a\) during the xylose consumption phase of the fermentation performance analysis performed in minimal medium with 50 g l\(^{-1}\) each of glucose and xylose.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Maximum specific xylose uptake rate (g h(^{-1}) gDW(^{-1}))</th>
<th>Ethanol (g gDW(^{-1}))</th>
<th>Xylitol (g gDW(^{-1}))</th>
<th>Glycerol (g gDW(^{-1}))</th>
<th>Acetate (g gDW(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMB3001-p426</td>
<td>0.09 ± 0.01(^b)</td>
<td>0.31 ± 0.00</td>
<td>0.32 ± 0.01</td>
<td>0.06 ± 0.00</td>
<td>0.02 ± 0.00</td>
</tr>
<tr>
<td>TMB3001-p6UDHA</td>
<td>0.09 ± 0.01</td>
<td>0.32 ± 0.01</td>
<td>0.30 ± 0.01</td>
<td>0.06 ± 0.00</td>
<td>0.02 ± 0.00</td>
</tr>
<tr>
<td>TMB3255-p426</td>
<td>0.05 ± 0.01</td>
<td>0.48 ± 0.01</td>
<td>0.11 ± 0.01</td>
<td>0.05 ± 0.00</td>
<td>0.04 ± 0.00</td>
</tr>
<tr>
<td>TMB3255-p6UDHA</td>
<td>0.03 ± 0.01</td>
<td>0.45 ± 0.00</td>
<td>0.13 ± 0.01</td>
<td>0.07 ± 0.06</td>
<td>0.02 ± 0.01</td>
</tr>
</tbody>
</table>

\(^a\)Average and deviations from duplicate experiments

Application of our previously developed evolutionary engineering procedure (31) to TMB3001-p6UDHA showed slower evolution of improved xylose catabolism under aerobic...
(Figure 1A) and microaerobic conditions, whereas, differently as with TMB3001 (31), no anaerobic growth could be achieved (data not shown). Together, these observations indicates that UdhA is transferring reducing equivalents in the undesired direction, from NADPH to NAD⁺, thus further increasing the redox problem generated by yeast xylose catabolism in TMB3255.

![Figure 1. Evolution of S. cerevisiae TMB3001 (boxes: A, B) (31), TMB3001-p6UDHA (triangles: C, D), and TMB3201-pTHHXT4 (circles: E, F) in aerobic carbon limited chemostat cultures at a D of 0.05 h⁻¹ on 1 g l⁻¹ glucose and 5 g l⁻¹ xylose (A, C, E), and on 5 g l⁻¹ xylose (B, D, F). The evolving population was subjected to EMS mutagenesis prior to inoculation of the chemostats. The steady state OD₆₀₀ (closed simbols), and residual xylose concentrations (open simbols) are shown.](image)
Evolutionary engineering of HXT4

Because of the absence of specific xylose transporters, xylose uptake in S. cerevisiae is mediated by facilitated diffusion through different glucose transporters (15). The affinity is therefore higher for glucose than for xylose, and xylose transport is inhibited until glucose is present in the medium. Thus, when xylose and glucose are present in paragonable amounts, like in some lignocellulose hydrolysates, efficient xylose transporters with low sensitivity to glucose would be desirable for fast xylose fermentation. For that purpose, we searched for mutant forms of the hexose transporter HXT4, which showed both, increased xylose uptake characteristics and lowered inhibition by glucose. Since HXT4 is a moderately low affinity glucose transporter with the best xylose transport characteristics (15), this transporter protein is the closest to the desired final mutant feature, and was therefore expected to evolve faster to the desired characteristics.

To generate mutated forms of HXT4 with improved xylose transport abilities, we subjected the S. cerevisiae strain TMB3201-pTHHXT4 to a shorter version of the chemostat-based evolution procedure (31). TMB3201 was not able to transport glucose, and therefore also xylose, because of the deletion of all 18 hexose transporters present in S. cerevisiae. Thus, the expressed HXT4 gene provides the sole possibility to transport both, glucose and xylose in TMB3201 (15). Like for TMB3001, TMB3201-pTHHXT4 improved gradually its aerobic xylose metabolism first on 1 g l⁻¹ glucose plus 5 g l⁻¹ xylose, and subsequently on sole 5 g l⁻¹ xylose (Figure 1B). The latter strain, however, achieved only an OD₆₀₀ of about 3.5 and a residual xylose concentration of 2.3 g l⁻¹ after 80 generation of selection on glucose plus xylose, compared to the values of 6.5 and 1.2 g l⁻¹, respectively, previously observed for TMB3001 (31). The following aerobic selection on sole xylose was able to further increase the steady state OD₆₀₀ from 2.3 to 3, decreasing the residual xylose concentration to 1 g l⁻¹. This selection procedure was terminated after both aerobic selection steps were completed, and 35 single clones of the resulting population were picked from sole xylose minimal medium plates. These clones were then screened for improved aerobic growth rate on sole xylose minimal medium in 96 deep well-plates in duplicate experiments. All picked clones grew at least more than twice as fast as the parent strain TMB3201-pTHHXT4 (Figure 2). In particular, clones Nr.1 and 4 achieved a maximal growth rates of about 0.12 and 0.1 h⁻¹, respectively, compared to the value of 0.015 h⁻¹ detected for the parent strain. In order to determine if the aerobic growth rate improvements were due to mutations in HXT4, we re-isolated the plasmids from clones Nr. 1 and 4 and sequenced the open reading frame of HXT4.
However, no mutations could be found in both re-isolated \textit{HXT4} genes, indicating that the xylose metabolism improvements are either caused by intracellular metabolic mutations or by an increased overexpression level of \textit{HXT4} but not by mutations in the protein coding sequence.

\textbf{Figure 2}. Maximum aerobic growth rates on sole xylose of 35 single clones that were isolated after generation 110 of the TMB3201-pTHHXT4 evolution experiment shown in figure 1. Averages and deviations of two duplicate experiments are shown. C: TMB3201-pTHHXT4.
DISCUSSION

Limited capacity of the pentose phosphate pathway, redox imbalance generated during anaerobic yeast xylose catabolism, and non-optimal xylose transport have been reported to be among the major bottlenecks for efficient xylose catabolism (29). Thus, we addressed these problems singularly using rational metabolic and evolutionary engineering alone or in combination.

We report here functional expression of the soluble transhydrogenase from *E. coli* UdhA in *S. cerevisiae*. Growth of a phosphoglucone isomerase-deficient mutant on glucose upon UdhA overexpression allowed us to estimate the pentose phosphate capacity in *S. cerevisiae* of 2.94 mMol gDW⁻¹ h⁻¹. Since the recombinant strain TMB3001 has been reported to show a maximum pentose phosphate pathway flux of 1.2 mMol gDW⁻¹ h⁻¹ during xylose catabolism alone (36), we conclude that the pentose phosphate pathway capacity is not limiting during anaerobic xylose catabolism in TMB3001, which agrees perfectly with a previous report (22). This pathway could, however, constitute a bottleneck if an optimal xylose fermentation efficiency has to be achieved, as it will probably become a relevant limiting factor when the primary major bottlenecks of xylose catabolism in TMB3001 are relived (e.g. redox imbalance).

Expression of UdhA in the xylose fermenting strain TMB3001 and its oxidative pentose phosphate pathway-negative mutant TMB3255 was not able to solve the redox-balancing problem generated by the different cofactor usage of xylose reductase and xylitol dehydrogenase. On the contrary, physiological analysis indicated that the transhydrogenase rather formed even more NADH through NADPH re-oxidation, which is the reverse of what is needed for efficient operation of anaerobic yeast xylose catabolism. Confirmation of this observation was provided by the inability to achieve anaerobic growth on sole xylose by evolutionary engineering (31) of an UdhA expressing TMB3001 strain. Overexpression of the soluble transhydrogenase from *Azotobacter vinelandii* in *S. cerevisiae* during anaerobic growth on glucose has been reported to result in a similar phenotype, which was ascribed to an unfavorable NADPH/NAD⁺ vs. NADH/NAD⁺ ratio (24). Thus, we suggest that this ratio is thermodynamically not favorable toward NADH re-oxidation and NADPH production in xylose catabolizing *S. cerevisiae*, even when the major NADPH producing pathway, the oxidative pentose phosphate pathway, is inactivated.

Although we could successfully evolve improved xylose-metabolizing mutants that depend exclusively on *HXT4* for sugar transport, no mutations could be identified in the *HXT4* coding
Therefore, improved aerobic xylose catabolism in the isolated mutants appears to be due to either increased transcription level of $HXT4$ or to other metabolic mutations. According to the results of the differential gene expression analysis performed on the superior xylose fermenting strain C1, the hexose transporter of choice for evolutionary engineering driven improvement would have been $GAL2$ (chapter 1). Since, however, this transporter was shown to mediate xylose uptake worse than $HXT4$ (15), and the evolution experiments reported here were started long before the DNA chip analysis results sorted, we used for this experiment the probably non optimal transporter $HXT4$. This demonstrates again the power of evolutionary engineering in retrieving suitable targets for improvement, which are difficult to find by rational approaches.

Acknowledgments

We thank Professor Bärbel Hahn-Hägerdal for sharing *S. cerevisiae* TMB3001 and TMB3255 with us, and Professor Eckhard boles for ENY.WA-1A, EBY44, TMB3201, and the plasmids p425, p426, and pTHHXT4. This work was supported by the Swiss Bundesamt für Bildung und Wissenschaft (BBW) within the European Commission Framework V.
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Metabolic engineering of a phosphoketolase pathway for pentose catabolism in *Saccharomyces cerevisiae*

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SUMMARY

Low ethanol yields on xylose hamper successful establishment of an economically viable bioprocess for ethanol production from hemicellulose rich plant material with *Saccharomyces cerevisiae*. A major obstacle is the limited capacity of yeast for anaerobic re-oxidation of NADH. Net re-oxidation of NADH could potentially be provided through the phosphoketolase pathway that channels carbon fluxes toward ethanol formation. By heterologous expression of phosphotransacetylase and acetaldehyde dehydrogenase in combination with the native phosphoketolase, we installed a functional phosphoketolase pathway in the xylose-fermenting *Saccharomyces cerevisiae* strain TMB3001c. The resulting 25% higher ethanol yield was achieved by the reduced accumulation of the by-product xylitol. The corresponding flux through the phosphoketolase pathway was about 30% of the optimum that would be required to eliminate xylitol and glycerol accumulation completely. Further overexpression of phosphoketolase, however, increased acetate accumulation and reduced consequently the fermentation rate. By combining the phosphoketolase pathway with the *ald6* mutation for reduced acetate formation, a strain with 20% higher ethanol yield and 40% higher xylose fermentation rate than its parent was engineered.
INTRODUCTION

Pentose-rich hemicellulose is a major constituent of abundant plant material that may serve as a cheap substrate for ethanol production (34). Unfortunately, the preferred microorganism for alcoholic fermentation, the yeast *Saccharomyces cerevisiae*, is not naturally capable to metabolize pentoses. Intense research in the past decade focused on metabolic engineering of yeast for catabolism of the pentoses xylose and arabinose (3, 10, 14, 21) to develop economically viable processes from non-starch substrates (27). For xylose catabolism, a new pathway was installed by overexpression of the *Pichia stipitis* NAD(P)H-dependent xylose reductase and NAD"-dependent xylitol dehydrogenase, in combination with the homologous xylulokinase (6, 13). The different co-factor preferences in the oxidoreductases create, however, an anaerobic redox balancing problem that manifests itself in the accumulation of the reduced intermediate xylitol and low ethanol yields. Engineering of redox metabolism such that xylose reductase uses higher fractions of NADH (1, 15) has been demonstrated to alleviate xylitol formation and the low ethanol yield, but the fermentation rate decreased. This was hypothesized to be caused by lowered NADPH-dependent xylose reduction (15). Accordingly, increased cytosolic NADPH formation caused improved xylose fermentation rate and higher xylitol yield at the expense of ethanol formation (2). Unless a different pathway is used for initial xylose utilization (11), redox metabolism must thus be engineered such that enough NADH is re-oxidized to increase the ethanol yield without concomitantly decreasing NADPH production that is needed to drive the xylose reductase reaction at a high rate (22, 26).

One possibility would be the phosphoketolase pathway that produces two and consumes three moles of NADH per mole of xylose converted to ethanol (Figure 1). Phosphoketolases (E.C. 4.1.2.9) are key enzymes of many lactic acid and bifidobacteria (9); converting xylulose-5-P to acetyl-P and glyceraldehyde-3-P, and/or fructose-6-P to acetyl-P and erythrose-4-P (17, 20). Since net one mole of NADH is re-oxidized for each mole of xylose, glycerol and xylitol accumulation may be reduced (Figure 1). This bacterial pathway would potentially allow maximal theoretical conversion of xylose to ethanol with a yield of 0.51 g ethanol per g xylose, without affecting the NADPH/NADH usage ratio of xylose reductase in yeast.

We describe here metabolic engineering of a functional phosphoketolase pathway in the xylose-fermenting *S. cerevisiae* strain TMB3001c (6) that expresses xylose reductase, xylitol dehydrogenase, and xylulokinase from a chromosomal integration. While low level xylulose-
5-P phosphoketolase activity was already reported in *S. cerevisiae* (7), the introduction of heterologous phosphotransacetylase and acetaldehyde dehydrogenase (acylating) was necessary to establish a functional pathway. During the course of this work we realized that already low acetate accumulation has a negative influence on the fermentation rate of xylose. By combining reduced acetate accumulation in an *ald6* mutant (5) with the phosphoketolase pathway, we engineered a strain with increased fermentation rate and ethanol yield.

**Figure 1.** Partial bioreaction network of the *S. cerevisiae* central carbon metabolism on the carbon source xylose. The bold arrows indicate the phosphoketolase pathway. PK: phosphoketolase, PTA phosphotransacetylase, ACDH: acetaldehyde dehydrogenase (acylating), ACH1: acetyl-CoA hydrolase, ALDx: aldehyde dehydrogenase isoenzymes. Hydrolysis of acetyl-P can occur at ATPases, e.g. the H⁺-ATPase (30). Extracellular metabolites are written in capitals.
MATERIAL AND METHODS

Strains, media and cultivation conditions

The *S. cerevisiae* strains used here are listed in table 1. Cultures were stored in aliquots supplemented with 15% (v/v) glycerol at -80°C and were revived by growth in YPD medium (10 g l\(^{-1}\) yeast extract, 20 g l\(^{-1}\) peptone, and 20 g l\(^{-1}\) glucose). The fermentation performance of all strains was compared in minimal medium (24) with 50 g l\(^{-1}\) each of glucose and xylose. Ethanol-dissolved ergosterol (Fluka) and Tween 80 (Sigma) were added to final concentrations of 0.01 g l\(^{-1}\) and 0.42 g l\(^{-1}\), respectively. The pH was maintained above 4.5 by adding 100 mM citric acid buffer (pH 5.5). Cultures were grown in 175 ml serum bottles, filled with 150 ml medium and stirred magnetically at 100 rpm and 30°C. CO\(_2\) accumulation was prevented by penetrating the rubber septum with a needle (0.45 x 10 mm).

### TABLE 1. Strains used

<table>
<thead>
<tr>
<th>Strains and populations</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMB3001c</td>
<td>CEN.PK113-1C (*MATa; leu2-3,112; trp1-289; ura3-52, his3-Δ1, MAL2-8c, SUC2), <em>his3::YlpXR/XDUfXK</em></td>
<td>(6)</td>
</tr>
<tr>
<td>TMB3001c-p6XFP</td>
<td>TMB3001c expressing phosphoketolase</td>
<td>This study</td>
</tr>
<tr>
<td>TMB3001c-p5EHADH2</td>
<td>TMB3001c expressing acetaldehyde dehydrogenase</td>
<td>This study</td>
</tr>
<tr>
<td>TMB3001c-p4PTA</td>
<td>TMB3001c expressing phosphotransacetylase</td>
<td>This study</td>
</tr>
<tr>
<td>TMB3001c-p4PTA/p5EHADH2</td>
<td>TMB3001c expressing phosphotransacetylase and acetaldehyde dehydrogenase</td>
<td>This study</td>
</tr>
<tr>
<td>TMB3001c-p6XFP/p4PTA/p5EHADH2</td>
<td>TMB3001c expressing phosphoketolase, phosphotransacetylase and acetaldehyde dehydrogenase</td>
<td>This study</td>
</tr>
<tr>
<td>TMBaLD6c</td>
<td>TMB3001c <em>ald6::KanMX</em></td>
<td>This study</td>
</tr>
<tr>
<td>TMBaLD6c-p4PTA/p5EHADH2</td>
<td>TMBaLD6c expressing phosphotransacetylase and acetaldehyde dehydrogenase</td>
<td>This study</td>
</tr>
</tbody>
</table>
Analytical methods

Cell growth was monitored by following the optical density at 600 nm (OD\textsubscript{600}). Cellular dry weight (DW) was determined from at least three 2 ml culture aliquots that were centrifuged at 13,500 rpm for 20 min in pre-dried and pre-weighed micro-centrifuge tubes, washed once with water, and dried at 110°C for 24 h to constant weight. Commercially available kits were used for enzymatic determination of glucose (Beckman, Fullerton, CA), xylose (Medichem, Steinenbronn, Germany), xylitol (R-Biopharm, Darmstadt, Germany), acetate (R-Biopharm), and glycerol (Sigma). Ethanol was determined by GC as was described before (23).

Determination of physiological parameters and intracellular metabolic fluxes

Maximum exponential growth rates on glucose were determined by log-linear regression of OD\textsubscript{600} versus time with growth rate (\(\mu\)) as the regression coefficient. The specific biomass yield (\(Y_{x/s}\)) was determined as the coefficient of linear regression of biomass concentration (DW) versus substrate concentration (S) during the exponential growth phase on glucose. The biomass concentration was estimated from OD\textsubscript{600}-to-DW correlations determined for each culture after glucose depletion (40-60 h after inoculation) and prior to the termination of the experiment (100-120 h after inoculation). Ethanol, xylitol, acetate, and glycerol yields on glucose or xylose were calculated by linear regression of by-product versus substrate concentrations during exponential growth on glucose or after glucose depletion up to the end of the experiment, respectively. The maximum specific xylose uptake rate was determined as the ratio of the maximal xylose concentration decrease slope to the average biomass concentration after glucose depletion and the end of the experiment. The amount of evaporated ethanol during fermentation performance analyses was estimated by following the ethanol concentration decrease in an identical fermentation set-up containing 100, 50, and 25 g l\textsuperscript{-1} ethanol solutions in the same minimal medium. An evaporation constant of 0.001 h\textsuperscript{-1} was determined.

The previously reported stoichiometric model (29) was used to estimate intracellular carbon fluxes during the xylose consumption phase of the above anaerobic batch fermentations. To estimate the fluxes through the phosphoketolase pathway, the corresponding pathway was implemented in the model as a single reaction that converts xylulose-5-P and NADH to glyceraldehyde-3-P, acetaldehyde, and NAD\textsuperscript{+}. To maintain a determined system of linear equations (25), the cofactor usage ratio of the xylose reductase
was assumed to remain unaltered. This assumption is supported by unaltered rates of xylose uptake and acetate formation upon installing the pathway. The flux to ethanol was defined as a free flux, whose computed values were compared with the experimentally determined ethanol production rates. The computed free fluxes were always within 14% of the experimental values, thus confirming the reliability of the employed stoichiometric model.

Molecular biology procedures

The ald6 (YPL061W) mutant of TMBALD6c, was generated with the homolog flanking region approach (28). Briefly, the kanMX4 cassette of the yeast strain Y02767 (BY4741; MATa, his3-A1, leu2-A0, met15-A0, ura3-A0, YPL061W::kanMX4) from the gene deletion project (31) was PCR-amplified with primers that were complementary to sequences 500 bp upstream (5’-gacaaaagaaaaacgaccgaaagg-3’) and downstream (5’-atatgatctctgtgacgcaaatgg-3’) of the deleted gene. The PCR product was used directly to transform TMB3001c with the lithium acetate method (8). Transformants were confirmed by PCR using each of the above primers in combination with the corresponding kanMX4 specific primer KanB: 5’-ctgcagcgagggccgtaat-3’ or KanC: 5’-tgattttgtagagccgctaa-3’.

The D-xylulose-5-P/D-fructose-6-P phosphoketolase gene xfp from Bifidobacterium lactis was cloned as an EcoRI - HindIII fragment from the vector pFPK5 (17) under the control of the constitutive, truncated HXT7 promoter of the p426 (URA3) plasmid (12) to yield p6XFP. Similarly, the Entamoeba histolytica acetaldehyde dehydrogenase gene EhADH2 was cloned as a BamHI - XbaI fragment from pET/EhADH2 (32) into the BamHI-SpeI-digested p425 (LEU2) plasmid (12) to generate p5EHADH2. Finally, the phosphotransacetylase gene pta was amplified from genomic DNA of Bacillus subtilis RB50:PRF69 (19) using the forward primer: 5’-cgggatccatggcagatttattttcaacagtg-3’ and the reverse primer: 5’-ccatcgcgtgatccgagctgacagttttcaagctg-3’. The resulting PCR product was ligated into the BamHI – Clal-digested p424 (TRP1) plasmid (12) to obtain p4PTA. For comparative physiological analysis, all strains contained three plasmids, with or without insert, so that supplementation with amino acids was not necessary.

Phosphoketolase activity assay

Cell extracts were prepared from mid-exponential growth phase cultures at an OD600 of about 1 in minimal medium with 20 g l⁻¹ glucose or 5 g l⁻¹ galactose plus 20 g l⁻¹ xylose. Cell pellets were harvested by centrifugation, washed twice with deionized water, and resuspended
in 50 mM Histidine-HCl buffer (pH 7.0), containing 20 mM KH₂PO₄/Na₂HPO₄ (pH 7.0), 2 mM dithio-threitol, and 1 mM MgSO₄. The suspension was vortexed with glass beads (0.5 mm diameter) at 4°C for 5 min, incubated on ice for 5 min, and vortexed again for 5 min. Cell debris and glass beads were separated by centrifugation at 20,000 x g and 4°C for 5 min. In vitro activity of D-xylulose-5-P phosphoketolase was determined by measuring the acetyl-P formed after addition of ribose-5-P that was converted to xylulose-5-P by the endogenous ribose-5-P isomerase and ribulose-5-P epimerase in crude extracts. For this purpose, we incubated 60 μl of cell extract containing 0.6 mM thiamine pyrophosphate and 6 mM D-ribose-5-P at 30°C for 10 min. The reaction was stopped by boiling for 5 min and centrifugation at 20,000 x g and 4°C for a further 5 min. The resulting supernatant was supplemented with 5 U of phosphotransacetylase and incubated at 30°C for 5 minutes to convert all acetyl-P to acetate. The acetate produced in the assay mixture was then determined enzymatically by subtracting the acetate that was formed in an assay mixture without ribose-5-P (blank) from the assay mixture containing ribose-5-P. The total protein content was determined with a commercially available kit (Beckman). Specific activities were expressed as units per mg of protein, where one unit is defined as formation of one micromole of acetate per min.
RESULTS

Installing a phosphoketolase pathway

To engineer the phosphoketolase pathway from xylulose-5-P to ethanol in the xylose fermenting *S. cerevisiae* strain TMB3001c (Figure 1), we expressed different combinations of the *B. lactis* D-xylulose-5-P/D-fructose-6-P phosphoketolase gene *xfp*, the *B. subtilis* phosphotransacetylase gene *pta*, and the *Entamoeba histolytica* acetaldehyde dehydrogenase gene *EhADH2*. During the initial glucose consumption phase in batch cultures with 50 g l\(^{-1}\) each of glucose and xylose, the biomass yields were about 30% lower in all phosphotransacetylase expressing strains (data not shown). Beside a slightly higher acetate yield in TMB3001c-p6XFP/p4PTA/p5EHADH2, all other parameters were very similar to those of the control strain. During the subsequent xylose consumption phase, phosphoketolase expressing strains exhibited all strongly reduced specific xylose uptake rates and xylitol yields (Figure 2). The most prominent change in the physiology of the phosphoketolase expressing strains was the extremely high accumulation of acetate. While separate expression of acetaldehyde dehydrogenase or phosphotransacetylase had only negligible effects, concomitant overexpression of both genes surprisingly increased the ethanol yield by about 25% when compared to the control strain TMB3001c (Figure 2). This was achieved by decreased xylitol formation but at an unaltered high uptake rate of xylose.

Since heterologous expression of phosphoketolase was apparently not necessary to improve the ethanol yield, we hypothesized endogenous phosphoketolase activity in *S. cerevisiae* as was described earlier during growth on xylose (7). To verify this endogenous phosphoketolase activity we used an *in vitro* enzyme assay. During exponential growth on glucose as the sole carbon source, a low activity was observed in the control strain, and significant induction was apparent during growth on galactose plus xylose (Table 2). Heterologous overexpression of phosphoketolase further increased the phosphoketolase activity about ten-fold in TMB3001c-p6XFP/p5EHADH2/p4PTA. The apparent induction of the recombinant phosphoketolase on galactose plus xylose may be explained by the moderate downregulation of the truncated *HXT7* promoter on glucose (12).
Figure 2. Physiological parameters of *S. cerevisiae* TMB3001c expressing various combinations of the phosphoketolase pathway genes during the xylose consumption phase (30 to 120 h) of anaerobic batch experiments on 50 g l\(^{-1}\) glucose and 50 g l\(^{-1}\) xylose. The average and deviation of two independent experiments is displayed.
TABLE 2. Specific xylulose-5-P phosphoketolase activity in *S. cerevisiae* expressing different combinations of the phosphoketolase pathway genes during growth on glucose or galactose plus xylose.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Carbon source</th>
<th>Specific activity (mU (mg protein)^(-1))</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMB3001c</td>
<td>Glucose</td>
<td>1.5 ± 0.1a</td>
</tr>
<tr>
<td></td>
<td>Galactose + xylose</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>TMB3001c-p4PTA/p5EHADH2</td>
<td>Glucose</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Galactose + xylose</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>TMB3001c-p6XFP/p4PTA/p5EHADH2</td>
<td>Glucose</td>
<td>13.5 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>Galactose + xylose</td>
<td>26.8 ± 7.8</td>
</tr>
</tbody>
</table>

*The average and the deviation were determined from two independent experiments.

During the xylose consumption phase, the flux through the phosphoketolase pathway in TMB3001c expressing phosphotransacetylase and acetaldehyde dehydrogenase was 45 μMol g^-1 h^-1 or about 6% of the xylose uptake rate (Figure 3). While this flux-rerouting clearly increased the ethanol yield, maximum theoretical ethanol production would require about three-fold higher phosphoketolase flux to eliminate glycerol and xylitol accumulation. The attempt to increase flux through the phosphoketolase pathway by additional heterologous overexpression of the phosphoketolase resulted in high acetate accumulation (Figure 2). Hence, we expressed all three phosphoketolase pathway enzymes in the ald6 mutant TMBALD6c that lacks the cytosolic constitutive aldehyde dehydrogenase, which is principally responsible for acetate production from acetaldehyde (5, 18). The acetate yield could be reduced to only about 0.23 g g^-1 by this strategy, indicating that the acetate formed upon phosphoketolase overexpression derives from the hydrolysis of either acetyl-P (30) or acetyl-CoA (4). This result leads to the conclusion that higher activities of either phosphotransacetylase or acetaldehyde dehydrogenase may be necessary to install a fully functional phosphoketolase pathway upon increased phosphoketolase activity. Additionally, deletion of genes like *ACH1*, coding for acetyl-CoA hydrolase that catalyzes the hydrolysis of acetyl-CoA (4), may further help in attaining this goal.
Figure 3. Specific carbon fluxes (μMol gDW⁻¹ h⁻¹) during the xylose consumption phase of anaerobic batch experiments on 50 g l⁻¹ glucose and 50 g l⁻¹ xylose. Fluxes for TMB3001c (upper values) and TMB3001c-p4PTA/p5EHADH2 (middle values) were calculated from the experimental data shown in Figure 2. The lower values (italics) represent the flux distribution for maximum theoretical ethanol from the experimental data of TMB3001c when no glycerol and xylitol production was assumed to be absent. Fluxes are average values of duplicate experiments with deviations within 10%.
Metabolic engineering of reduced acetate formation improves xylose catabolism

The strong decrease of xylose fermentation rate observed upon phosphoketolase overexpression (Figure 2), but also upon deletion of the glucose-6-P dehydrogenase-encoding ZWF1 (15), were accompanied by a drastically higher accumulation of acetate. Thus, we investigated the effect of the extracellular acetate concentration on the xylose fermentation behavior in yeast. We compared the fermentation performance of TMB3001c and TMBALD6c in anaerobic batch culture with 50 g l⁻¹ of each glucose and xylose. During growth on glucose, acetate formation of TMBALD6c was below the detection level, whereas TMB3001c accumulated up to 1.5 g l⁻¹ acetate (data not shown). During xylose consumption, TMBALD6c still produced much less acetate but exhibited a 50% higher specific xylose uptake rate than TMB3001c (Table 3).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Specific xylose uptake rate (g g⁻¹ h⁻¹)</th>
<th>Yields on xylose (g g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ethanol</td>
</tr>
<tr>
<td>TMB3001</td>
<td>0.12</td>
<td>0.33</td>
</tr>
<tr>
<td>TMB3001 + acetate</td>
<td>0.07</td>
<td>0.36</td>
</tr>
<tr>
<td>TMBALD6c</td>
<td>0.18</td>
<td>0.32</td>
</tr>
<tr>
<td>TMBALD6c + acetate</td>
<td>0.12</td>
<td>0.35</td>
</tr>
</tbody>
</table>

*The deviation of duplicate experiments was below 10% of the mean.

*The deviation of duplicate experiments was below 5% of the mean.

*The acetate concentration increased by 2 g l⁻¹ upon glucose depletion.

To demonstrate that the higher rate of xylose consumption was indeed caused by the reduced acetate accumulation, we increased the acetate concentration immediately after glucose depletion from 0 and 1.5 g l⁻¹ to 2 g l⁻¹ and 3.5 g l⁻¹ for TMBALD6c and TMB3001c, respectively. Consequently, specific xylose uptake rates were significantly reduced in both strains, and TMBALD6c exhibited about the same rate as TMB3001c without the addition of acetate (Table 3). These results provide evidence that acetate has a negative effect on the xylose fermentation rate.
Establishment of the phosphoketolase pathway in TMBALD6c

A simultaneous increase of ethanol yield and xylose fermentation rate would clearly be desirable. Hence, we engineered the phosphoketolase pathway into the ald6 mutant by expression of the acetaldehyde dehydrogenase and phosphotransacetylase in TMBALD6c. While the resulting strain TMBALD6c-p5EHADH2/P4PTA maintained the high ethanol yield of TMB3001c-p5EHADH2/P4PTA, it exhibited very low acetate production and a 40% increased specific xylose uptake rate (Figure 4). Thus, both useful characteristics could be combined without significant loss of the previously observed improvements.
Figure 4. Physiological parameters of TMBALD6c and TMBALD6c-p4PTA/p5EHADH2 during the xylose consumption phase of anaerobic batch experiments on 50 g l⁻¹ glucose and 50 g l⁻¹ xylose. TMB3001c and TMB3001c-p4PTA/p5EHADH2 (white bars) data from Figure 2 are shown for comparison. The average and deviation of two independent experiments is displayed.
DISCUSSION

We describe here the successful establishment of a functional phosphoketolase pathway in a recombinant xylose-consuming *S. cerevisiae* strain. In combination with the endogenous phosphoketolase activity (7), heterologous expression of phosphotransacetylase and acetaldehyde dehydrogenase was sufficient to achieve significant flux through this novel pathway. This metabolic engineering strategy improved the ethanol yield on xylose by about 25%, without affecting the xylose fermentation rate. Maximal theoretical yield calculation indicated, however, that the flux through the phosphoketolase pathway should be three-fold higher for optimal ethanol production from xylose. Attempting to increase the flux through heterologous overexpression of the phosphoketolase from *B. lactis* resulted in strong acetate accumulation. This is probably explained by insufficient phosphotransacetylase or acetaldehyde dehydrogenase activities. In this case, acetate could be formed by the hydrolysis of either acetyl-P (30) or acetyl-CoA (4).

Somewhat independently of the phosphoketolase pathway, we found that acetate was a strong inhibitor of xylose fermentation in yeast. Deletion of the NADPH-dependent aldehyde dehydrogenase-encoding *ALD6* gene strongly reduced acetate formation, thereby increasing the rate of xylose fermentation in TMBALD6c by about 50% when compared to TMB3001c. Notably, the ethanol yield remained largely unaltered. Since strains with increased ethanol yield *and* xylose fermentation rate are important for commercial establishment of an ethanol production process, we attempted to combine both metabolic engineering strategies: reduction of acetate formation and the phosphoketolase pathway establishment. Expression of the two phosphoketolase pathway enabling enzymes phosphotransacetylase and acetaldehyde dehydrogenase in the low acetate producer TMBALD6 yielded in a strain, which fermented xylose about 40% faster and produced ethanol with a 20% higher yield than TMB3001c.

Metabolic engineering strategies of xylose metabolism in yeast led often to either increased ethanol yields at the expenses of xylose consumption rate (1, 15) or increased fermentation rates by lowering the ethanol yield (2, 33). Two notable exceptions were reported recently. In the first, ammonium assimilation was modified by deleting the NADPH-dependent glutamate dehydrogenase *GDH1* and overexpressing the NADH-dependent isoenzyme *GDH2* (22). Consequently, the xylitol yield was decreased by 45% causing an ethanol yield increase and a concomitant xylose fermentation rate increase by 15%. Since this NADH re-oxidizing reaction is linked to anabolism, however, the possibilities to further improve the corresponding flux to completely eliminate xylitol and glycerol accumulation
appear to be limited. In the second case, xylose reductase expression levels were increased in a zwf1 mutant with an interrupted pentose phosphate pathway (16). In the optimal combination, the ethanol yield was increased by 10% and the fermentation rate by 120%. The drawback of this approach was a 150% increased glycerol production, since xylose reductase catalyzes also the conversion of dihydroxyacetone-P to glycerin-3-P. In contrast to the above two approaches, the phosphoketolase pathway is an alternative catabolic pathway that channels the carbon fluxes directly to ethanol, without interfering with other natural metabolic processes. Since this pathway consumes more NADH than it requires for the conversion of xylose to ethanol, it appears to be a promising metabolic engineering strategy for further improved ethanol production from xylose. To fully accomplish this duty, the enzymatic activities of phosphoketolase, phosphotransacetylase, and acetaldehyde dehydrogenase need probably to be increased and fine tuned. Investigation of the concomitant disruption of the acetyl-CoA ACH1 gene may be of interest for contributing to decrease the acetate accumulation upon phosphoketolase overexpression.

Acknowledgments

We thank Bärbel Hahn-Hägerdal for sharing S. cerevisiae TMB3001c with us, Jörg Hauf for Y02767, Leo Meile for providing the xfp gene from B. lactis, Samuel L. Stanley Jr. for the EhADH2 gene from E. histolytica, and Eckhard Boles for the plasmids p424, p425, and p426. This work was supported by the Swiss Bundesamt für Bildung und Wissenschaft (BBW) within the BIO-HUG project of the European Commission Framework V.
REFERENCES


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Fermentation performance of engineered and evolved xylose-fermenting *Saccharomyces cerevisiae* strains

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Lignocellulose hydrolysate is an abundant substrate for bioethanol production. The ideal microorganism for such a fermentation process should combine rapid and efficient conversion of the available carbon sources to ethanol with high tolerance to ethanol and to inhibitory components in the hydrolysate. A particular biological problem are the pentoses, which are not naturally metabolized by the main ethanol producer *Saccharomyces cerevisiae*. Several recombinant and evolved xylose fermenting *S. cerevisiae* strains have been developed recently. We compare here the fermentation performance and robustness of eight recombinant strains and two evolved populations on glucose/xylose mixtures in defined and lignocellulose hydrolysate-containing medium. Generally, the polyploid industrial strains depleted xylose faster and they were more resistant to the hydrolysate than the laboratory strains. The industrial strains accumulated, however, up to 30% more xylitol and produced therefore less ethanol than the haploid strains. The three most attractive strains were the selected, extremely rapid xylose consumer TMB3400, the evolved C5 strain with the highest achieved ethanol titer, and the engineered industrial F12 strain with the by far highest robustness to the lignocellulosic hydrolysate.
INTRODUCTION

The renewable energy source bioethanol is readily obtained by fermentation of starch-containing agricultural products such as sugar cane juice or cornstarch. Since feedstock costs are the most critical issue for commercial competition with fossil fuels (20), intense efforts have focused on lignocellulose-based bioprocesses (6, 24). These abundant polymers are major components of agricultural, paper, and starch industry waste streams that would be economically relevant raw materials for low-cost ethanol production. Different from starch-based feedstocks, however, lignocellulosic hydrolysates contain not only glucose, but also a variety of sugars.

The yeast Saccharomyces cerevisiae is used since millennia by mankind for high performance ethanol production from hexose sugars. This microorganism exhibits high resistance to ethanol, fermentation by-products, and other inhibiting substances that are present in lignocellulose hydrolysates (6, 11). Since S. cerevisiae is incapable of fermenting pentoses, suboptimal ethanol production is obtained from hemicellulosic hydrolysates with their high pentose contents (6). Consequently, metabolic engineering has been used extensively in the past decade to establish and improve catabolism of the pentoses arabinose (2, 12) and in particular xylose (3, 6, 8). Over-expression of the Pichia stipitis xylose reductase and xylitol dehydrogenase, and of the endogenous xylulokinase resulted only in limited success, since the achieved xylose fermentation rates and ethanol yields were low in comparison to those achieved on glucose (3, 7, 9, 18). Nevertheless, the strategy is useful because it improved final ethanol titers of a polyploid industrial S. cerevisiae strain on mixtures of glucose and xylose (23). Further S. cerevisiae strains of laboratory or industrial background with significantly improved xylose fermentation properties were recently isolated from evolutionary (15) or selection strategies (22). Since all those reports focused on particular aspects of xylose metabolism and on one or few strains, it is difficult to evaluate the capabilities of the presently available variants.

The focus of this work was quantitative comparison of the fermentation performance of the available laboratory and industrial strains on xylose/glucose mixtures in defined minimal medium and in lignocellulose hydrolysate medium.
MATERIAL AND METHODS

Strains and media

All *S. cerevisiae* strains used are listed in table 1. Into *Saccharomyces cerevisiae* F, xylose reductase and xylitol dehydrogenase from *P. stipitis* were introduced and endogenous xylulokinase was overexpressed, using the procedures described in (23). Several clones were isolated and F12 was selected as described previously (23), as the most efficient clone. Strains were stored at -80°C in culture aliquots containing 15% glycerol and were routinely plated on YPD plates (10 g l⁻¹ yeast extract, 20 g l⁻¹ peptone, 20 g l⁻¹ glucose, and 1.5% (w/v) technical agar (Becton Dickinson)). Before initiating physiological experiments, cultures were grown once in the minimal medium at pH 5.5 (19) using 20 g l⁻¹ glucose as carbon source. For anaerobic cultivation, the medium was supplemented with ergosterol (Fluka) and Tween 80 (Sigma). Both components were dissolved in boiling 99.8% (v/v) ethanol and were added to the medium at a final concentration of 0.01 g l⁻¹ and 0.42 g l⁻¹, respectively. Lignocellulose hydrolysate for growth inhibition studies and fermentation performance analysis was obtained by a two-step dilute-acid hydrolysis of Norway spruce (*Picea abies*), as described previously (10). The particular hydrolysate chosen was rather toxic, even to a robust microbe like *S. cerevisiae*.

Cultivation conditions

The fermentation performance of all strains was compared in minimal medium with 50 g l⁻¹ each of glucose and xylose. The concentrations of all minimal medium components except KH₂PO₄ were doubled. To avoid major drops in pH, 100 mM citric acid buffer (pH 5.5) was added, which maintained the pH above 4.6 in all cases. Cultures were grown in 175 ml serum bottles, filled with 150 ml medium and stirred magnetically at 100 rpm and 30°C. To establish anaerobic conditions, each bottle was sparged for 10 min with pure N₂ (O₂ < 5 ppm) (PanGas, Dagmersellen, Switzerland) prior to inoculation. CO₂ accumulation was prevented by penetrating the rubber septum with a needle (0.45 x 10 mm).
## TABLE 1. Strains and populations used

<table>
<thead>
<tr>
<th>Strains and populations</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Laboratory strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMB3001</td>
<td><em>S. cerevisiae</em> CEN.PK 113-7A (MATα, his3-Δ1, MAL2-8c, SUC2) his3::YipXR/XDH/XK</td>
<td>(3)</td>
</tr>
<tr>
<td>Evolved TMB population</td>
<td>Evolved population obtained through evolutionary engineering of TMB3001</td>
<td>(15)</td>
</tr>
<tr>
<td>C1</td>
<td>Clone isolated from the evolved TMB population</td>
<td>(15)</td>
</tr>
<tr>
<td>C5</td>
<td>Clone isolated from the evolved TMB population</td>
<td>(15)</td>
</tr>
<tr>
<td><strong>Industrial strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>Industrial, polyploid <em>S. cerevisiae</em> strain</td>
<td>Gift from Bruno Jarry</td>
</tr>
<tr>
<td>F12</td>
<td><em>S. cerevisiae</em> F HIS3::YIploxZEO overexpressing XR, XDH, and XK</td>
<td>This study</td>
</tr>
<tr>
<td>A4</td>
<td><em>S. cerevisiae</em> A HIS3::YIploxZEO (Industrial, polyploid strain) overexpressing XR, XDH, and XK</td>
<td>(23)</td>
</tr>
<tr>
<td>Evolved A4 population</td>
<td>Evolved population obtained through evolutionary engineering of A4</td>
<td>This study</td>
</tr>
<tr>
<td>BH42</td>
<td>Strain with improved xylose catabolism obtained by breading</td>
<td>(16)</td>
</tr>
<tr>
<td>TMB3399</td>
<td><em>S. cerevisiae</em> USM21 HIS3::YIpXR/XDH/XK (Industrial, polyploid strain) overexpressing XR, XDH, and XK</td>
<td>(22)</td>
</tr>
<tr>
<td>TMB3400</td>
<td>Isolated after mutagenesis and selection of TMB3399</td>
<td>(22)</td>
</tr>
</tbody>
</table>

The minimal inhibitory concentration of lignocellulosic hydrolysate was determined by adding increasing concentrations to 20 g l\(^{-1}\) glucose-containing minimal medium. These 50 ml cultures were incubated in 250 ml shake flasks at 140 rpm and 30°C. The minimal inhibitory concentration was defined as the lowest hydrolysate concentration that prevented exponential growth for at least 48 h after inoculation from a freshly streaked YPD-plate (10).
Fermentation performance of selected strains on lignocellulose hydrolysate was compared in 800 ml, in house-built fermentors at 30°C and 250 rpm without sparging of any gas. A constant pH of 5.5 was maintained by automatic addition of 3 M KOH to a culture volume of 750 ml. The medium contained 10% (v/v) lignocellulosic hydrolysate in a minimal medium containing triple amounts of salts, vitamins, and trace elements, and the following final concentrations of sugars were: 22 g l⁻¹ glucose, 9 g l⁻¹ xylose, 16 g l⁻¹ mannose, 4 g l⁻¹ galactose and 2 g l⁻¹ arabinose. The medium was supplemented with 0.05% (v/v) antifoam (Dow Corning® Antifoam RD Emulsion, BDH Laboratory Supplies, Poole, UK), 0.03 g l⁻¹ ergosterol and 1.2 g l⁻¹ Tween 80.

Chemostat selection was performed as described previously on minimal medium in a Sixfors 6-minireactors system (Infors, Botmingen, Switzerland) at a dilution rate (D) of 0.05 h⁻¹ and a working volume of 300 ml (15). Microaerobic conditions were installed by gradually decreasing aeration over a period of 10 generations until complete shut-off of aeration at generation 70. Finally, anaerobic conditions were achieved by slight sparging (< 1 ml min⁻¹) with technical N₂ (<200 ppm of O₂). It should be noted that due to the contaminating O₂, these conditions were not strict anaerobic.

**Analytical methods**

Cell growth was monitored by following the optical density at 600 nm (OD₆₀₀). Cellular dry weight (DW) was determined by filtering 10 ml of culture broth through a pre-weighted 0.45µm filter and washing with 30 ml of water. The filter was dried in a microwave oven at 350 W for 8 min, cooled in a desiccator, and weighed.

Commercially available kits were used for enzymatic determination of glucose (Beckman, Fullerton, CA), xylose (Medichem, Steinenbronn, Germany), xylitol (R-Biopharm, Darmstadt, Germany), acetate (R-Biopharm), and glycerol (Sigma) in pure minimal medium fermentations. During these cultivations, ethanol was determined by GC as described before (14). Substrates and products in lignocellulose hydrolysate containing fermentations were measured by refractive index column liquid chromatography (CLC). To measure the concentrations of glucose, arabinose, xylitol, glycerol, acetate, and ethanol, we used two Aminex HPX-87H columns (Bio-Rad, Hercules, CA) operating in series at 45°C, with sulphuric acid (5 mM) at a flow rate of 0.5 ml min⁻¹ as the mobile phase. Water at a flowrate of 0.5 ml/min was the mobile phase for the Aminex HPX-87P column (Bio-Rad, Hercules,
CA) operating at 80°C, that was used to determine xylose, galactose, and mannose concentrations.

**Determination of physiological parameters**

Maximum specific growth rates were determined by log-linear regression of OD$_{600}$ versus time with growth rate ($\mu$) as the regression coefficient. The specific biomass yield ($Y_{\text{biomass/s}}$) was determined as the coefficient of linear regression of biomass concentration (DW) versus substrate concentration (S) during the exponential growth phase on glucose. The biomass concentration was estimated from OD$_{600}$-to-DW correlations determined for each culture immediately after glucose depletion, and immediately before termination of the experiment.

During the exponential growth phase on glucose, the specific glucose uptake rate was calculated as the ratio between $\mu$ and $Y_{\text{biomass/Glucose}}$. The maximum specific xylene uptake rate was determined as the ratio of the maximal xylene concentration decrease slope to the average biomass concentration between glucose depletion and the end of the experiment. Ethanol, xylitol, acetate, and glycerol yields on glucose or xylose were calculated by linear regression of by-product concentration versus substrate concentration during exponential growth on glucose or after glucose depletion until the end of the experiment, respectively. The amount of evaporated ethanol during the fermentation performance analyses was estimated by following the ethanol concentration decrease in an identical fermentation set-up containing 100, 50, and 25 g l$^{-1}$ ethanol solutions in the same minimal medium (21). An evaporation constant of 0.001 h$^{-1}$ was determined.
Chapter 6

RESULTS

Fermentation performance in minimal medium

Anaerobic fermentation of a mixture of 50 g \text{l}^{-1} xylose and 50 g \text{l}^{-1} glucose in minimal medium was used to evaluate the performance of eight strains including the laboratory strain TMB3001, the entire evolved TMB population, two clones of the population (C1 and C5), the industrial strains F12, A4, TMB3399, and the selected or bread derivatives thereof (TMB3400 and BH42) (Figure 1). All strains contain chromosomal copies of the three genes that are required for xylose utilization (3). As expected from its impaired glucose metabolism (15), the evolved laboratory strain C1 was by far the slowest during the first growth phase on glucose (Figures 1 and 2). While glucose catabolism generally preceded xylose catabolism, C1 exhibited a significant period of xylose co-metabolism (Figure 1). Upon glucose depletion, all strains ceased to grow and the rate of catabolism slowed down significantly (Figure 1). Nevertheless, the evolved or selected strains C1, BH42, and TMB3400 strains exhibited significantly higher maximum specific rates of xylose consumption than the other strains (data not shown). This indicates that these strains were selected for an improved property of xylose metabolism that has not yet been recognized, and thus was not part of the present metabolic engineering strategy. Overall, the industrial F12 and TMB3400 strains consumed xylose most rapidly. In particular TMB3400 fermented all available carbon within 60 h, about half the time that was required by most other strains (Figure 1A). Although TMB3400 was clearly the most rapidly fermenting strain, it produced only a final ethanol titer of about 36 g \text{l}^{-1} ethanol, which was similar to the titer of most other strains, while the slower C5 strain achieved the highest ethanol titer of more than 39 g \text{l}^{-1} (Figure 1C). This superior ethanol production of C5 resulted primarily from the high yield of ethanol and the low formation of the side-product xylitol during the xylose consumption phase (Figure 3).
Figure 1. Fermentation process performance of *S. cerevisiae* strains in minimal medium supplemented with 50 g l⁻¹ each of glucose and xylose. Time course of xylose (A), glucose (B), ethanol (C), and biomass (D) concentrations of one of two duplicate experiments is shown. TMBEP: evolved TMB population.
To obtain more detailed metabolic insights, we then compared the yields of biomass and products during the two phases of glucose and xylose consumption (Figure 3). With the exception of BH42, the polyploid industrial strains generally produced more biomass and less acetate when compared to the haploid laboratory strains during exponential growth on glucose. From xylose, the industrial strains produced less ethanol and more of the undesired side-product xylitol, when compared to TMB3001 and its derivatives, C1, C5, and the evolved TMB population. Notably, the C1, BH42, and TMB3400 strains that were isolated upon selection for improved xylose catabolism, diverted significantly more of the carbon flow from either glucose or xylose to the reduced by-product glycerol. This provides strong evidence that those strains contain mutations that affect redox metabolism.

![Figure 2](image_url)

**Figure 2.** Maximum specific growth rates of *S. cerevisiae* strains during anaerobic growth on glucose. Average values and deviations of duplicate experiments are shown. TMBEP: evolved TMB population.
Figure 3. Biomass and product yields of *S. cerevisiae* strains on glucose (left column) and on xylose (right column) during fermentation in minimal medium containing 50 g l\(^{-1}\) each of xylose and glucose. Average values and deviations of duplicate experiments are shown. TMBEP: evolved TMB population.

**Long-term chemostat evolution of the industrial A4 strain**

Clearly no single variant combined all desirable features for rapid *and* efficient ethanol production, and the generally more rapid industrial strains suffered from high xylitol and low ethanol yields on xylose. Since the previously developed multi-step chemostat evolution procedure (15) successfully improved the xylose metabolism of the laboratory strain TMB3001, as evidenced by the C1 and C5 strains (Figures 1 and 3), we applied the same evolution procedure to the industrial strain A4. In contrast to TMB3001, A4 could grow aerobically on sole xylose in chemostat culture at a dilution rate of 0.05 h\(^{-1}\). Thus, it was not
necessary to first evolve this capacity, that took about 90 generations for TMB3001 (15). After gradual improvement of aerobic xylose metabolism for about 60 generations (Figure 4A), microaerobic conditions were installed for further 330 generations (Figure 4B). Aeration of the reactor was completely stopped at generation 70, which led to a shift from aerobic to microaerobic conditions within one generation. Generally, microaerobic evolution of the polyploid A4 strain was much slower than was observed previously for the haploid TMB3001 (15). After a total of 390 generations, the evolved A4 population grew to a steady state OD$_{600}$ of 2.2 at a residual xylose concentration of 1 g l$^{-1}$ under microaerobic conditions, which was similar to the TMB3001 population under the same conditions. The evolved A4 population, however, was washed out upon installing anaerobiosis by sparging the chemostat with technical N$_2$ (data not shown), while the evolved TMB3001 population was apparently able to tolerate the complete absence of oxygen and continued to propagate at a rate of 0.05 h$^{-1}$ in the previously reported experiments (15). After the comparatively long evolution process, the final evolved A4 population exhibited a one third higher maximum specific xylose consumption rate than the A4 parent (Figure 5), whereas the yields of ethanol and xylitol remained identical (data not shown). These changes in physiological performance are similar to those observed for the evolved TMB population (Figure 3). Since A4 exhibited already a one third higher specific xylose uptake rate than TMB3001, the evolved A4 population had a 75% higher rate than TMB3001, 0.21 g g$^{-1}$ h$^{-1}$. This improvement brings the evolved A4 population close to the best-performing industrial strains TMB3400 and BH42. Physiological analysis of eight clones from the evolved A4 population revealed that no clone was a significantly better or faster ethanol producer than the population (data not shown).

![Figure 4](image)

**Figure 4.** Evolution of the industrial *S. cerevisiae* A4 strain in carbon-limited chemostat culture at a D of 0.05 h$^{-1}$ in 5 g l$^{-1}$ xylose minimal medium under aerobic conditions (A), and under microaerobic conditions (B). Steady-state OD$_{600}$ (●) and xylose concentration (○) are shown. The evolving population was subjected to EMS mutagenesis prior of the inoculation of the chemostat.
Fermentation in the presence of lignocellulose hydrolysate

Realistic process conditions entail the presence of cheap, unpurified, and typically hydrolyzed plant material (4, 6). Hence, we determined the minimum dilute-acid spruce hydrolysate concentration that prevents aerobic growth of TMB3001, C1, C5, TMB3399, TMB3400, F12, and BH42 on glucose for at least 48 h after inoculation (10) (Table 2). The particular hydrolysate chosen is relatively toxic, even to a robust microorganism like S. cerevisiae (10). While the haploid laboratory strains TMB3001, C5, C1, and the hybrid strain BH42 were rather sensitive as found previously for laboratory strains (5), the polyploid industrial strains TMB3399 and TMB3400 exhibited a modest resistance. By far the most resistant strain was F12, with a minimum inhibitory concentration of about 45% (v/v) of hydrolysate.

Since only TMB3399, TMB3400, and F12 grew in minimal medium supplemented with 10% (v/v) lignocellulose hydrolysate, we compared their fermentation performance under those conditions. The following pure substrates were supplemented to obtain a composition

![Graph](image-url)
akin to that typically found in undiluted lignocellulose hydrolysate (10): 22 g l\(^{-1}\) glucose, 9 g l\(^{-1}\) xylose, 4 g l\(^{-1}\) galactose, 2 g l\(^{-1}\) arabinose, and 16 g l\(^{-1}\) mannose. Although all three strains attained the same final titer of ethanol, TMB3400 consumed xylose again more rapidly than the two other strains (Figure 6). Perhaps as a consequence of its enhanced metabolism, TMB3400 accumulated the highest concentration of the undesired by-products glycerol and acetate (Table 3). As would be expected from the higher resistance, F12 achieved the highest biomass concentration. Notably, arabinose was quantitatively converted to arabitol in all strains (data not shown).

TABLE 2. Minimum inhibitory concentrations of lignocellulose hydrolysate in glucose minimal medium batch cultures for some industrial and laboratory strains.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Minimal inhibitory hydrolysate concentration (% (v/v))</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMB3001</td>
<td>10</td>
</tr>
<tr>
<td>C1</td>
<td>10</td>
</tr>
<tr>
<td>C5</td>
<td>10</td>
</tr>
<tr>
<td>TMB3399</td>
<td>15</td>
</tr>
<tr>
<td>TMB3400</td>
<td>15</td>
</tr>
<tr>
<td>F12</td>
<td>45</td>
</tr>
<tr>
<td>BH42</td>
<td>10</td>
</tr>
</tbody>
</table>

TABLE 3. Substrate and product concentrations achieved by F12, TMB3399 and TMB3400 after 71 h fermentation on minimal medium containing 10% (v/v) lignocellulose hydrolysate\(^a\).

<table>
<thead>
<tr>
<th>Strains</th>
<th>Glucose (g l(^{-1}))</th>
<th>Xylose (g l(^{-1}))</th>
<th>Galactose (g l(^{-1}))</th>
<th>Arabinose (g l(^{-1}))</th>
<th>Mannose (g l(^{-1}))</th>
<th>Xylitol (g l(^{-1}))</th>
<th>Glycerol (g l(^{-1}))</th>
<th>Acetate (g l(^{-1}))</th>
<th>Ethanol (g l(^{-1}))</th>
<th>Biomass (g l(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>22</td>
<td>9</td>
<td>4</td>
<td>2</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>0.4</td>
<td>1.8</td>
<td>0</td>
</tr>
<tr>
<td>F12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.3</td>
<td>0</td>
<td>2.3</td>
<td>3.4</td>
<td>0.4</td>
<td>17.6</td>
<td>4.3</td>
</tr>
<tr>
<td>TMB3399</td>
<td>0</td>
<td>1.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3.6</td>
<td>3.0</td>
<td>0.6</td>
<td>17.7</td>
<td>3.8</td>
</tr>
<tr>
<td>TMB3400</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2.7</td>
<td>4.6</td>
<td>1.0</td>
<td>17.7</td>
<td>3.7</td>
</tr>
</tbody>
</table>

\(^a\) Values are the averages from duplicate experiments with deviations below 10% of the mean if not displayed.
Figure 6. Fermentation performance of the industrial background *S. cerevisiae* strains F12 (▲), TMB3399 (■), and TMB3400 (★) in minimal medium containing 10% (v/v) lignocellulose hydrolysate. Time courses of xylose (A), glucose (B), ethanol (C), and biomass (D) concentrations of one of two duplicate experiments is shown.
DISCUSSION

We demonstrate here that desirable xylose fermentation parameters were significantly improved in the *S. cerevisiae* strains Cl, C5, TMB3400, and BH42 compared to their respective parent strain/s. Thus, random methods of selection, breading and evolutionary engineering (13) were pivotal to the improvement of particular process properties. Clearly, the choice of the starting strain(s) for such random procedures is critical, and the polyploid industrial isolates were more resistant to the presence of lignocellulose hydrolysate. Overall, the mutagenized and selected polyploid strain TMB3400 (22) appears to be most promising because it was by far the most rapid in converting sugars to ethanol, reducing the total fermentation time from the typical 90-150 h to below 60 h. Moreover, it was somewhat resistant to the presence of hydrolysate. These properties would lead to commercially attractive short fermentation periods. Two other potentially interesting strains were the evolved haploid laboratory strain C5 (15), which accumulated the highest ethanol titer but lacked the desired robustness, and the industrial F12 strain that was by far the most resistant to the hydrolysate.

The industrial strains accumulated generally more of the undesired by-product xylitol, which in turn decreased their ethanol yield on xylose. While xylitol formation was much lower in the evolved TMB3001-derivatives C1 and C5, their useful properties cannot be exploited directly for industrial ethanol production because they lacked robustness. Breading and selection for improved xylose catabolism generated the hybrid mutant BH42, which exhibited, together with C1, the highest aerobic growth rate on sole xylose among all strains studied (data not shown). However, the robustness feature was not maintained in this strain.

The successful improvement of desirable process properties from various strains by different random approaches suggests that further improved process strains will be generated in this fashion. Methods of evolutionary engineering (13) appear to be most promising for initial introduction of a novel (15) or for improvement of a weak property (17). A straight forward approach would be to select or screen for strains with improved robustness to lignocellulose hydrolysate. Combining the best properties of the currently available strains could potentially be achieved by enhanced breading techniques (25) or through the identification of the molecular basis of certain desirable features and inverse metabolic engineering (1).
Acknowledgments

We thank Bruno Jarry, Orsam-Amylum, France, for kindly providing the strain *S. cerevisiae*. Ana Borges, Center for Process Biotechnology, DTU is thanked for construction of F12 and Christophe Roca, Center for Process Biotechnology, DTU is thanked for characterization of this strain. Katja Franke is thanked for technical assistance. This research was funded within the European Commission Framework V project BIO-HUG.
REFERENCES


Evolutionary engineering of *Escherichia coli* for increased metabolic activity in stationary phase

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SUMMARY

Increased metabolic activity in nutrient starvation-induced stationary phase has potential to improve productivity of metabolites or proteins by increasing the operational life-span of bioprocesses and decoupling production from growth. We describe here selection procedures for the evolution of *Escherichia coli* strains with increased catabolic rates in nitrogen starvation-induced stationary phase. The best selection procedures for the enrichment of such mutants were found to be nitrogen-limited continuous cultures with a very low dilution rate of 0.03 h\(^{-1}\). After 40 generations of selection, improved mutants with up to doubled catabolic rates in stationary phase were isolated. Screening for clones with highest catabolic rates was performed with a novel procedure, wherein the metabolic activity was assessed by measuring the specific glucose uptake rates in nitrogen starvation-induced stationary phase in the 96 well format.
INTRODUCTION

Evolutionary engineering of useful microbial traits has often been used when limited understanding of complex phenotypes precluded rational strategies (22), and recent examples include improved resistance to environmental stresses (24, 28), enhanced production capabilities (27, 30), and novel catabolic activities (1, 23). One such biotechnologically interesting but complex phenotype is high metabolic activity in the absence of growth. Under quiescent conditions, energetic and nutritional resources could then potentially be channeled toward product formation, such that the organism becomes a true catalyst that does not waste resources for replicating itself.

This phenotype is already present in higher multicellular organisms, where cells sometimes differentiate by entering a quiescent status with an elevated metabolic activity. A characteristic example is provided by the differentiation of hepatocytes during the maturation of the postnatal liver. During this process, the acquisition of a complete spectrum of all hepatic functions coincides with reversible cell cycle arrest in the G1 phase (8). Enhanced protein productivity in the absence of growth could be engineered in CHO cells by expressing the cyclin-dependent-kinase inhibitor p27 that allowed growth arrested and improved production for at least seven days (16). A similar phenotype could be obtained in mammalian cells also by a temperature shift from 37°C to 30°C (11). In Escherichia coli, which is still a preferred host for protein production, protein production during cell cycle arrest was successfully achieved by expressing the Rcd RNA (regulator of cell division) in a H-NS (histone-like nucleoid structuring protein) deficient mutant (20). In this final case, however, a continuously decreasing production phase of only 10 h was reported after growth arrest induction.

For the economically viable production of metabolites and proteins with low market and high raw material prices, high production rates that are maintained for long time periods are necessary. Thus, further work to improve the bioconversion capabilities of Escherichia coli in the absence of growth is necessary, and nutrient starvation induced stationary phase is an attractive condition where uncoupling of production from growth could be achieved. Moreover, higher metabolic activities in stationary phase are of additional interest because of the potential to increase the operative live-span of already established fermentation processes for the production of primary metabolites (15).

In bacteria, the reaction to nutrient shortage is characterized by the complex interaction between metabolism and other cellular processes to maintain viability under conditions not
propitious for growth (5, 9, 12, 18). Upon nutrient depletion, the biotechnologically relevant enteric bacterium *Escherichia coli* enters stationary phase, which is characterized by a metabolically inactive state with increased resistance to environmental stresses (7, 26). In *E. coli*, this physiological transition has been the subject of numerous studies, revealing a complex and still not fully elucidated regulation network (6, 29) that is activated already before the growth rate slows down (10, 17). The carbon-starvation response is regulated by the interplay of different intracellular regulator molecules, wherein ppGpp and cAMP are thought to play central roles (29). These molecules are involved in the regulation of the principal stationary phase and stress response regulator, the alternative transcription factor σ^S_5, which is coded by *rpoS* (6, 13). The biotechnologically most important consequence of stationary phase induction is the general decrease in metabolic rates.

The complex and not fully elucidated regulation of stationary phase in *E. coli* heavily hampers metabolic engineering of this organism for the direct improvement of its metabolic activity after nutrient starvation. Similarly, the exploitation of evolutionary engineering is hampered by the fundamental problem to identify selection conditions that allow the propagation of cells but enrich for those that are more active in the absence of growth. Hence, we test here different selection conditions to evolve mutants with increased metabolic rates in stationary phase. For rapid screening of improvement, we developed a system with high-throughput potential for the determination of the specific glucose uptake rate in stationary phase.
MATERIAL AND METHODS

Strains and media

We used the *Escherichia coli* strains MG1655 (2), CA8000 (25), and SP850 (CA8000 Δ*cyb*: *kan*) (25) throughout this study. The strain suffixes CNL refer to clones that were isolated from the nitrogen-limited continuous culture selection after 40 generations. Stocks for strain maintenance were generated from overnight cultures grown in LB medium (10 g l⁻¹ tryptone, 5 g l⁻¹ yeast extract, 10 g l⁻¹ NaCl) by adding glycerol to a final concentration of 15% (w/v), and were stored at -80°C. To preserve the original clonal composition of selection chemostats, aliquots of the populations from the selection cultures were frozen directly without intermediate batch growth. Prior to evolution or physiological experiments, we grew the cultures once overnight in minimal medium (pH 7.0), containing per litre: 19 mM NH₄Cl, 8.5 mM NaCl, 53 mM Na₂H₂PO₄, 22 mM KH₂PO₄, 0.1 mM CaCl₂, 2 mM MgSO₄, 1.3 μM thiamine, 0.6 mM FeCl₃, 6.3 μM ZnSO₄, 7 μM CuCl₂, 7.1 μM MnSO₄, 7.6 μM CoCl₂, 0.6 mM EDTA. For aerobic shake flask cultivation, we grew 50 ml cultures in 500 ml baffled shake flasks, which were incubated at 37°C and 200 rpm. Solid media were prepared by adding 1.5% (w/v) technical agar (Becton Dickinson) to LB or minimal medium. Finally, culture sample dilution for single clone isolation from minimal medium plate, was done with saline solution (150 mM NaCl, 10 mM MgSO₄).

EMS mutagenesis

To increase genetic variability, an LB culture of MG1655 was randomly mutagenized with ethyl methane sulfonate (EMS). For this purpose, we washed twice a 5 ml culture with LB (5,000 rpm and 4°C for 5 min) as soon as an OD₆₀₀ of 0.5 was achieved. The resulting pellet was re-suspended in 2.5 ml LB, and 35 μl EMS were added. After 2h of incubation at 30°C, we washed the cells again twice with 5 ml LB. The final pellet was re-suspended in 2 ml LB, and stored at -80°C after addition of glycerol. With this procedure, a survival rate of about 10% could be achieved.

Chemostat selection

Chemostat cultures used for selection purposes were performed in 2 l stirred tank reactors (Bioengineering, Wald, Switzerland) at a dilution (growth) rate of 0.03 h⁻¹ and a culture volume of 1 l. A pH of 7.0 was maintained by automatic addition of 2 N NaOH, and the
temperature was set at 37°C. Culture mixing and aeration were performed at 1000 rpm and 11 min⁻¹, respectively. Discontinuous feeding profiles at D 0.03 h⁻¹ were achieved by addition of 5 ml medium each 6 minutes via an automatic dispenser (Biospectra, Schlieren, Switzerland). Constant volume was maintained by automatic weight control (Bioengineering). We used minimal medium containing a reduced amount of 34 mM NaH₂PO₄ and 5 g l⁻¹ glycerol for carbon-limited chemostat cultures. To achieve nitrogen limitation, minimal medium with the same reduced phosphate content, only 6.5 mM NH₄Cl, and an increased glycerol content of 15 g l⁻¹ was used. To avoid foam formation, polypropylene glycol P2000 was added to a final concentration of 0.1% (v/v) in the culture medium.

**Screening method**

To screen for clones with improved specific glucose uptake rate in stationary phase, single clones were grown in 96 deep well-plates incubated in a rotary shaker at 30°C and 300 rpm (3). In this setup, each single clone was grown in four separate cultures of 1 ml nitrogen-limited minimal medium containing differently from the standard composition: 9 mM NH₄Cl, 100 mM 2-(N-morpholino)-ethanesulfonic acid (MES) buffer (pH 7.0), and 15 g l⁻¹ glucose. Culture samples were collected starting from 10 h after inoculation at intervals of 5-15 h until 50 h from the first sample withdrawal. OD₆₀₀ and glucose concentrations were measured in 96-well plate (E.I.A./R.I.A flat bottom plate, Costar, XYZ) format using a SpectraMaxPlus plate reader photometer (Molecular Devices, Sunnyvale, CA) and a commercially available enzymatic kit for glucose determination (Böhringer Mannheim).

**Analytical methods**

Cell growth was monitored by following the optical density at 600 nm (OD₆₀₀). Cellular dry weight (DW) was determined from 10 ml culture aliquots that were centrifuged at 5,000 rpm for 20 min in pre-weighed glass tubes, washed once with water, and dried at 110°C for 24 h to constant weight. Commercially available kits were used for enzymatic determination of glucose (Beckman) and glycerol (Sigma).

**Determination of physiological parameters**

In batch culture, exponential growth rates were determined by log-linear regression of OD₆₀₀ versus time with growth rate (μ) as the regression coefficient. The specific biomass yield was determined as the coefficient of linear regression of biomass versus glucose
concentration during the exponential growth phase. The biomass concentration was estimated only for shake flask cultures from predetermined \( \text{OD}_{600} \)-to-DW correlations during the mid-exponential growth phase of aerobic cultures on glucose minimal medium. During the exponential growth phase the specific glucose uptake rate was calculated as the ratio between the growth rate and the biomass yield. During stationary phase, this value was determined as the ratio of the linear regression coefficient of glucose concentration versus time and the average biomass concentration during the same time interval. Since no \( \text{OD} \)-to-DW correlation was determined during clonal screening, the corresponding specific stationary phase glucose uptake rates were calculated using the average \( \text{OD}_{600} \) instead of the biomass concentration.
RESULTS

Screening for higher metabolic activity in stationary phase

Evolved populations are often heterogeneous for a defined phenotype, and a suitable and simple screening procedure is needed to identify single clones with superior improvements compared to the overall population average. Several analytical procedures based on colorimetric or fluorimetric plate assays were previously found to be not reliable for the identification of clones with high metabolic activities in the stationary phase (4). Here, we developed a new and robust method, which is based on liquid medium cultivation, and thus resembles as closely as possible industrially relevant conditions. Specifically, we grew quadruplicate cultures for each clone in nitrogen-limited minimal medium with glucose excess. To achieve sufficient throughput, cultures were grown under aerobic conditions in 96-deepwell-plates, which were previously shown to reliably mimic traditional shake flask cultures (3). After a short exponential growth phase of about 8 to 10 h, cultures achieved a nitrogen-starvation induced stationary phase at an OD_{600} of about 0.8 - 1.6 (Figure 1). During stationary phase (10 h - 50 h), the cultures continued to consume glucose at a constant rate and unaltered OD_{600}. It is thus reasonable to assume that cellular metabolism is in a quasi-steady state. As a measure of the catabolic rate, we determined the specific glucose uptake rate in stationary phase. Quadruplicate experiments typically resulted in a standard deviation of about 10%, confirming the reliability of this method.

Figure 1. Growth and glucose consumption of *E. coli* MG1655 in nitrogen-limited batch culture used for screening purposes. OD_{600}, closed circles; glucose concentration, open circles. Stationary phase is underplayed in gray. Error bars indicate error propagation from four parallel determinations.
Selection for increased metabolic activity in stationary phase

Perhaps the most critical factor in evolutionary engineering is the identification of a pertinent selection scheme. The selection procedure should subject the evolving population to a selection pressure which favors the overgrowth by mutants with the desired phenotype. In our case, however, the desired phenotype contradicts apparently the basic principle of growth-dependent selection, since we seek higher metabolic activities in a non-growing status. Thus, we attempted to identify conditions that give a selective advantage to such mutants.

In a first attempt, we grew three EMS-mutagenized populations of *E. coli* MG1655 in serial nitrogen-limited, batch cultivations using glucose, fructose, or glycerol as carbon sources, respectively. Our hypothesis was that cells with a more active metabolism in stationary phase would recover faster from this “dormant” state when re-inoculated in fresh medium, thus, exhibiting shorter lag-phases and a consequent growth head start. However, no increase in the specific glucose uptake rate in nitrogen starvation-induced stationary phase could be detected at the population or clonal level after eight re-inoculation steps that correspond to about 40 generations of selection (data not shown).

In a second set of experiments, we dramatically increased the frequency of the growth-starvation cycles by cultivating two EMS-mutagenized populations of MG1655 in glycerol-limited and an ammonium-limited chemostat cultures with a discontinuous feeding profile for 40 generations (40 days). To establish such feeding conditions, 5 ml of fresh culture medium were pulsed each 6 minutes to achieve cycles of maximum growth and starvation. Under these conditions, at each medium pulse, the limiting nutrient concentration in the culture was calculated to increase instantaneously from undetectable levels up to 0.1 and 0.3 mM for ammonium and glycerol, respectively. The added nutrient was completely consumed maximally two minutes after the pulse addition, so that cells could starve for at least four minutes. We hypothesized that cells that react faster to the nutrient supply would scavenge completely the low amount of nutrients that are added during the pulses, whereas the others would not scavenge sufficient substrates and would be washed out. Since the use of glucose in long-term selection chemostats has been previously shown to select for efficient co-metabolism (19), and results in the suppression of mutations (14), we grew both selection chemostats on glycerol, which circumvents these problems to some extent (14, 21). Like for the serial batch based selections, no increased specific glucose consumption could be observed at both, population and single clone level, in the glycerol-limited discontinuous chemostat culture. The nitrogen-limited culture, on the contrary, was able to enrich for
mutants with increased specific glucose uptake rates in stationary phase. Indeed, the analysis of selection culture aliquots at 0, 10, 20, 30, and 40 generations revealed a continuous increase in catabolic activity in the first 20 generations. This 50% improvement compared to MG1655 could, however, not be increased further in the following 20 generations (Figure 2A). Moreover, analysis of 12 clones isolated from the culture aliquot after 40 generations, showed that a significant number of clones exhibited the same improvement as the population, without, however, achieving higher values (Figure 2B).

![Figure 2](image_url). Specific glucose uptake rate in stationary phase measured for population aliquots at various stages of evolution (A, C) and for clones isolated from population aliquots at 40 generations (B, D). The ammonium-limited chemostat culture with discontinuous feeding profile (A, B), and the ammonium-limited continuous culture (C, D) are shown. C: MG1655; P40: population aliquot at 40 generations. Error bars indicate error propagation from four parallel determinations.

Higher frequencies of the growth-starvation cycles caused improved enrichment for the desired phenotype. Therefore, we selected two EMS mutagenized pools of MG1655 for about 40 generations in two parallel continuous feeding profile chemostat cultures at a dilution rate of 0.03 h\(^{-1}\). This extremely low value, which corresponds to the overall dilution rate of the discontinuous feeding profile chemostats, was chosen because stationary phase regulation signals increase with decreasing growth rate (17). The value of 0.03 h\(^{-1}\) was also the lowest that could be achieved with our fermentation setup. Carbon-limitation was established in the first, and nitrogen-limitation in the second culture. To assess the efficacy of the two culture conditions for the selection of improved catabolic rates in stationary phase, we measured the
specific glucose uptake rate in stationary phase of population aliquots during the entire selection process. Again, we found that only the nitrogen-limited conditions yielded populations with significantly increased catabolic rates (Figure 2C). After 40 generations, a nearly doubled rate compared to MG1655 could be observed for the nitrogen-limited (Figure 2C) chemostat, whereas no change was observed for the glycerol-limited culture (data not shown). In the first case, the population was quite homogenous, with an elevated number of clones showing a maximum increase of about 70 to 100%, which is about the same value observed for the population (Figure 2D).

Figure 3. Specific glucose uptake rates in stationary phase of clones isolated after 0 (A), 10 (B), 20 (C), and 40 (D) generations of selection of chemically mutagenized E. coli MG1655. For comparison, the wild type and the entire selected population at each step are plotted (darker bars). The hairline indicates a two-fold increased specific glucose uptake rate compared to MG1655. Error bars indicate error propagation from four parallel determinations.
To investigate the selection process in more detail, we isolated 12-16 single clones from culture aliquots at 0, 10, 20, and 40 generations of the nitrogen-limited continuous feeding profile chemostat culture. Screening for the specific glucose uptake rate in stationary phase revealed a steady enrichment profile of improved clones over the entire 40 generations selection (figure 3). Mutants with a doubled catabolic rate in stationary phase appeared already after 10 generations, and were rapidly enriched to become the major subpopulation after 40 generations. Vice versa, clones with an unchanged phenotype compared to MG1655 or with minor improvements decreased their frequency in the evolving population during selection.

Selection in a cAMP deficient background

The stationary phase specific alternative transcription factor σ^8 is regulated at transcriptional, translational and post translational level. In this complex regulation network, the carbon catabolite repression regulatory complex CRP-cAMP was found to act negatively, whereas the stringent response regulatory molecule ppGpp positively on σ^8 regulation. The overall role of these two actors in the starvation response of E. coli is, however, only indicative and not yet precisely defined. Because of its general regulatory function, negative mutants of cAMP and ppGpp might be potentially advantageous for the evolution of increased metabolic activity in stationary phase. Since ppGpp deficient mutants are not able to grow on minimal medium without amino acid supplementation (31), we could not use such mutants directly in the selection scheme. Hence, we selected only an EMS mutagenized population of the cAMP deficient strain SP850 under ammonium limitation in continuous chemostat culture for 40 generation. No significantly augmented specific glucose consumption increase was detected in nitrogen-limited stationary phase after 40 generations at the population level. The best single clone that we isolated from a 40 generations culture aliquot exhibited only about 30% improvement compared to the parent SP850, among a majority of clones with unchanged catabolic rates (Figure 4). These results indicate that the absence of cAMP negatively affects the selection process toward increased metabolic activity in stationary phase.
Shake flask cultures

To confirm the results obtained with the screening method, we cultivated the selected mutants CNL7, CNL9, CNL12, CNL14, and the parent MG1655 in nitrogen-limited batch cultures in shake flask. For each mutant we determined the DW to OD$_{600}$ correlation, and used the resulting stationary phase biomass concentration to calculate the corresponding specific glucose uptake rate. This experiment confirmed the same quantitative improvements previously detected with the screening method. The analyzed mutants consumed glucose both, absolutely and specifically, faster than MG1655 (partially shown in Figure 5). Moreover, they achieved an about 15% lower, but more stable stationary phase OD$_{600}$, which, differently from MG1655, showed no decline over the entire measured stationary phase of up to 80 h (data not shown). Finally, during the exponential growth phase, the analyzed mutants exhibited in average also increased growth rates and specific glucose uptake rates of about 15% (data not shown). Thus, indicating that the mutants improved their metabolic rate properties not only in stationary phase, but moderately also during exponential growth.
Figure 5. Specific glucose uptake rate during exponential growth (A) and stationary phase (B) of the parental MG1655 strain and four mutants isolated from the nitrogen limited continuous culture selection at 40 generations. The cultures were performed in shake flasks. Average values and deviations from two independent experiments are shown.
DISCUSSION

We investigated the efficiency of different selection schemes for the enrichment of *Escherichia coli* mutants with an increased specific glucose uptake rate in nitrogen-starvation induced stationary phase, which was taken here as an example for increased metabolic activity in stationary phase. Because this improved property is independent from growth, its achievement by mutagenesis and continuous growth-based selection is apparently paradoxical. We found that the most successful procedure for the selection of this improved phenotype was nitrogen-limited continuous culture with a dilution rate of 0.03 h\(^{-1}\) (Figure 2). The selection of MG1655 under these conditions for about 40 generations yielded an about 100% increased catabolic rate in stationary phase. Nitrogen-limited chemostat culture with a discontinuous feeding profile was significantly less efficient in this selection process, achieving only an about 50% improvement after 40 generations. On the contrary, no improvements were observed in both carbon-limited continuous and discontinuous feeding profile chemostat cultures, as well as in the nitrogen-limited serial batch selection procedures.

At sub-maximal growth rates caused by nutrient limitation, *E. coli* is in a metabolic state of hunger (5). At the onset of this particular physiological state, the cells begin to exhibit stationary phase induction. Nevertheless, they still need to remain metabolically active in order to scavenge trace amounts of the limiting nutrient, and mutants that scavenge the nutrients faster would therefore overgrow the others (5). Continuous cultures provide the possibility to constrain slow growth, and thus allow the long-term continuous induction of a hunger state in the evolving population, which would explain the efficacy of this selection scheme in selecting for higher metabolic activity in stationary phase. Moreover, the existence of different hunger states for each different limiting-nutrient type (5) explains why only nitrogen-limited continuous culture was able to improve the catabolic rate after nitrogen starvation in batch culture. In particular, the nitrogen regulatory protein C (NtrC) and the alternative transcription factor σ\(^{54}\) play specific central roles in the nitrogen-limitation caused hunger state and stationary phase induction (32), and may not be relevant under carbon starvation. Finally, the slower enrichment of mutants with increased metabolic activity in stationary phase in a cAMP negative population indicates that this intracellular metabolite is important also for the regulation of the nitrogen-limitation caused hunger state and stationary phase induction, as already observed under carbon limitations (5).

A strain that optimally exploits stationary phase for product formation would exhibit high metabolic activity for extended periods. In MG1655 the catabolic rate in stationary phase is
only about 8% of the rate during exponential growth (Figure 5). In the best mutant that we isolated from the nitrogen-limited continuous culture, this value climbs to 16%. Moreover, this improvement was maintained for at least 40 h after the onset of stationary phase (data not shown), that is considerably more than the about 10 h reported previously for a quiescent cell protein expression system (20). The achieved improvement is remarkable but probably not sufficient to establish an industrial process, indicating that further improvement of the evolutionary engineering strategy may be necessary.

The occurrence of spontaneous mutagenesis during the continuous selection process is very useful for the generation of superior phenotypes that are based on multiple mutations, and the probability that such mutants are already present in the starting population is very low. Hence, we analyzed the clonal composition during the progression of continuous selection in nitrogen-limited continuous culture to find indications for the appearance of new mutants generated by to spontaneous mutagenesis during the selection process (Figure 3). Clones with doubled catabolic rate in stationary phase could be detected already after 10 generations, and no mutants with further improved rates could be found even after 40 generations. Thus, it appears that the spontaneous mutagenesis rate was insufficient to generate more complex mutants with more than doubled catabolic rates during the short selection period of 40 generations. Only mutants that were present already in the starting population were enriched. The employment of mutator strains with a strongly increased spontaneous mutagenesis rate (24) might improve the evolutionary engineering strategy toward the selection of industrially more relevant phenotypes without extending excessively the timeframe of continuous selection.

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Conclusions

I present here the successful application of evolutionary engineering for the generation of the first known eukaryotic organism that is able to grow on sole xylose under strict anaerobic conditions (Chapter 2). This unique yeast strain has apparently overcome the principal bottlenecks of anaerobic xylose catabolism, which, in eukaryotes, is typically initiated with the two step oxidoreductase conversion of xylose to xylulose. We identified the low ATP production rate as the most likely fundamental limitation for anaerobic growth on sole xylose (Chapter 3). This bottleneck is caused by the limited NADH re-oxidation capacity under anaerobic conditions that then results in low glycolytic fluxes. Thus, strategies that aim to improve ethanol production from xylose using the two-step oxidoreductase pathway should focus on NADH re-oxidation.

Transhydrogenases could potentially provide NADH re-oxidation by simultaneously producing NADPH, which would be advantageous for optimal xylose reductase activity. We show here, however, that the soluble transhydrogenases catalyzes the reverse reaction in xylose fermenting yeast, producing even more NADH, probably for thermodynamic reasons (Chapter 4). Energy-dependent membrane-bound transhydrogenases would potentially be able to re-oxidize NADH independently from the relative concentrations of the two co-factor species. Hence, their expression needs to be targeted to a membrane with a correctly oriented proton gradient, which is then used to fulfill the otherwise thermodynamically unfavorable reaction. A first alternative to transhydrogenases is the establishment of a new pathway capable of net NADH re-oxidation. We demonstrated that this possibility can be realized by establishing the phosphoketolase pathway (Chapter 5). We showed that phosphoketolase activity is already present in yeast and that the expression of the remaining enzymes of the pathway, the phosphotransacyetylase and the acylating acetaldehyde dehydrogenase, is sufficient to improve the ethanol yield. Nevertheless, further engineering of this pathway is necessary to provide complete NADH re-oxidation. A second alternative is the modification of existing pathways to provide net NADH re-oxidation. For instance, this could be attained by expression of a NADPH-dependent glyceraldehyde-3-P dehydrogenase, which would eliminate the redox neutrality during ethanol production via glycolysis. Finally, the entire redox problem would be circumvented by expression of the bacterial pathway for xylose catabolism that is carried out by xylose isomerase. Until now, however, a xylose isomerase that allows higher ethanol productivities on xylose than those obtained with the eukaryotic oxidoreductase pathway, is not available.

Redox balancing is the principal problem of anaerobic xylose metabolism in yeast, but not the sole problem for optimal ethanol production from xylose. We suggest that xylose transport
may become a limiting factor because increased expression of xylose transport mediating proteins occurred in our best xylose-fermenting mutant (Chapter 3). Additionally, optimal ethanol productivities from lignocellulose hydrolysates require simultaneous fermentation of glucose and xylose. For that purpose an efficient xylose transporter that is not inhibited by glucose is needed. According to the genome-wide transcript levels and metabolic fluxes in our best mutant, xylose fermentation might be even further improved by increasing the activity of the xylose catabolic pathway itself, and by overexpressing genes of the pentose phosphate pathway, once the redox and transport problems are solved (Chapter 3).

Finally, the optimized xylose and arabinose catabolic pathways should be implemented in different industrial strains with high tolerance to inhibitors that are present in lignocellulose hydrolysates (Chapter 6). Subsequent rounds of evolutionary engineering and genome shuffling will probably generate even further improved strains for direct use in industrial ethanol production from hydrolyzed plant material.

In a somewhat independent part of this thesis, we found that long-term, nitrogen-limited continuous cultures are able to enrich for *Escherichia coli* mutants with increased metabolic activity in stationary phase under nitrogen starvation (Chapter 7). To achieve industrially relevant increased productivities of e. g. primary metabolites, the selection of a production strain with a transiently increased spontaneous mutagenesis rate for longer periods under the found conditions may be necessary. Transiently increased spontaneous mutagenesis rates could be achieved by the use of a curable plasmid overexpressing a mutated DNA polymerase with defective proofreading functions. These findings may be directly applicable also to ethanol production with yeast.
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