# Physiological responses of carbon fluxes to deletion of specific genes in Saccharomyces cerevisiae.

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# Chapter 8

# General Discussion

This thesis has dealt with the effect and control of some key enzymes in carbon metabolism and on the distribution of carbon fluxes over the fermentative and the oxidative route in *Saccharomyces cerevisiae*. Additionally, a method has been developed, by measuring intracellular metabolites, that can identify the function of silent genes, *i.e.* genes that have no effect on growth rate when deleted. This method can help to elucidate the function of unknown genes and contribute to the comprehension of the complex behaviour of yeast.

## Characterising silent genes

One of the ultimate goals of the yeast genome sequencing and functional analysis project is the elucidation of the function of all gene products of *S. cerevisiae*. Two thousand of the six thousand genes have an unknown function, whereas half of these genes reveal no obvious phenotype when they are deleted [47], which complicates their characterisation. In Chapter 2 we have tried to tackle this problem by developing two separate approaches which are both based on the accurate tracking of shifts in metabolite profiles in mutants deleted in a silent gene, compared to its parent. The methods were based on the idea that when the deletion of a gene encoding a functional product does not reveal an obvious phenotype, *e.g.*, in growth rate, the metabolite profile must have changed.

The approaches can be divided into a specific and a comprehensive method. The 'specific' method is based on the measurements of metabolite concentrations and subsequent co-response analysis. The comprehensive method is based on examination of a large, arbitrary set of metabolites by NMR techniques, providing 'metabolic snapshots'. Both methods were demonstrated to be successful. Therefore, it can be concluded that this simple method of measuring the metabolome is a powerful approach to elucidate the function of many of the silent genes in yeast and maybe even in man or other organisms.

## The contribution of $F_1 \cdot F_0$ ATP synthase to yeast physiology

Chapters 3 and 4 dealt with the 'splendid molecular machine', $F_1 \cdot F_0$ ATP synthase [24]. In both chapters the contribution of $F_1 \cdot F_0$ ATP synthase to carbon fluxes and energy metabolism was investigated and quantified. The contribution of $F_1 \cdot F_0$ ATP synthase was expected to be minimal under glucose repressing conditions. In Chapter 3, it becomes clear that $F_1 \cdot F_0$ ATP synthase plays an essential role in carbon and energy metabolism, also at high glucose.

Neglecting the strong dependence on $F_1 \cdot F_0$ ATP synthase for ATP supply on non-fermentable carbon sources, the intracellular ATP concentration was not affected by decreasing the $F_1 \cdot F_0$ ATP synthase concentration in *S. cerevisiae* (see Chapter 4). Addition of different concentrations of uncoupler to wild-type cells growing on ethanol/glycerol medium showed that growth could not be maintained as soon as intracellular ATP levels decreased. Either the yeast maintained ATP at wild-type levels and continued to grow (at reduced rate) or the ATP levels decreased and the yeast stopped growing immediately. These results are quite opposite to results obtained with *Escherichia coli* [96-99] where growth was barely affected by a decrease in ATP synthase activity but ATP/ADP ratios were strongly reduced. In mammals and insects, however, ATP homeostasis is a well-known phenomenon. The ATP demand can increase over a hundredfold without affecting the intracellular ATP concentration. Additionally, very recently it was found that in higher eukaryotes lowering of the intracellular pH is an early event in the mitochondrial pathway for apoptosis and that the cellular cell death could be prevented by inhibiting $F_1 \cdot F_0$ ATPase [132]. In Chapter 4, acidification of the cytoplasm has also been observed in yeast strains with reduced levels of $F_1 \cdot F_0$ ATPase activity. Further research on the relationship between ATP homeostasis and intracellular acidification (and possible cell death) deserves further investigation. This can be accomplished by either using uncouplers or using the strains with regulable expression of the $F_1 \cdot F_0$ ATPase activity. As has become clear in Chapters 3 and 4, *S. cerevisiae* seems to exert ATP homeostasis like higher eukaryotes. A very central question that needs to be addressed further is: what factor couples the ATP supply of the $F_1 \cdot F_0$ ATP synthase activity to the ATP consuming reactions leading to the observed ATP homeostasis in yeast. The answer to this question may help to elucidate this problem in higher eukaryotes as well.

Although the ATP homeostasis seems powerful, compartmentalisation may have an effect on the ATP concentrations measured in the whole cell extracts that have been used to determine ATP concentrations. It has not been excluded that compartment-specific ATP levels (*i.e.* in the mitochondria) have changed upon a change in $F_1 \cdot F_0$ ATP synthase and therefore still may be the signal that controls the growth rate, intracellular acidification etc.

## Crabtree negative properties of an *hxk2* deletion strain

Hexokinase II (*HXK2*) is the first enzyme in glycolysis and catalyses the conversion of glucose to glucose 6-phosphate. Additionally it plays a crucial role in the glucose repression pathway. Derepressed mRNA levels and protein activities of some glucose repressible genes showed that hexokinase II relieved glucose repression of several genes [50,53,127,128,137,251]. In spite of the discovery of this property more than 20 years ago, no extensive physiological characterisation of *hxk2* strains was reported. In Chapters 5A, 5B and 6 an extensive physiological characterisation of such a strain has been performed. Fully oxidative growth was observed on excess glucose, which is exceptional for a Crabtree positive yeast such as *S. cerevisiae* (see Chapter 5). As a result, the yield of biomass was strongly increased during exponential growth on glucose and ethanol production was below the detection level at early exponential phase and strongly reduced at late exponential growth phase. This radical change in physiological behaviour is scientifically interesting and the high yield and low ethanol production is also very appealing for industrial applications such as bakers' yeast production and heterologous protein production.

We have not been able to trace the agent causing the onset of ethanol production during the mid-exponential phase. Accordingly it is not excluded that the onset is caused by glucose signalling (*e.g. HXK1*) or by signalling, independent of glucose repression, causing an overflow of fluxes at the level of pyruvate towards ethanol. In Chapter 5A oxygen limitation and glucose could be excluded as possible triggers for the alcoholic fermentation. Nutrient limitations do not seem to play a role since the ethanol production of the *hxk2* deletion strain on rich medium was even more pronounced than on the defined medium (data not shown). It was observed consistently that the onset of alcohol production was related (indirectly) to biomass concentration. This makes it tempting to speculate that perhaps the onset of fermentation is related to the accumulation of a secondary metabolite that triggers alcoholic fermentation when it (and accordingly the cells) reaches a certain density. This can simply be tested by inoculating fully oxidatively growing *hxk2* deletion cells in the supernatant of a fermenting, high cell-density *hxk2* deletant culture, and analyse whether fermentation is triggered.

In Chapter 5B, an enhanced critical specific growth rate or oxidative capacity was demonstrated in glucose limited chemostat cultures for the *hxk2* deletion strain. Accordingly, an enhanced maximal growth rate was observed compared to the wild-type strain. An inversed relationship of the residual glucose concentration to the specific growth rate was observed both in batch and in continuous cultures, which could explain the difference in growth rate in batch (relatively high glucose) and glucose limited chemostat (low glucose). Possibly, the extracellular

glucose concentration dictates the glucose concentration in the *hxk2* deletant due to the relative low flux through glycolysis combined with the wild-type capacity of hexose transport in this strain. This would cause the intracellular glucose levels to become elevated at high extracellular glucose concentrations. An elevated intracellular glucose concentration can cause osmotic damage and/or unspecific O-glycosylation of proteins. The damage would grow worse with increasing levels of extracellular glucose what would explain the inverse relation between the specific growth rate and extracellular glucose concentration. However, this hypothesis has yet to be tested. It would be fascinating to find out how the transcriptome, proteome and metabolome have changed in the *hxk2* deletion strain in chemostat cultures to achieve a higher growth rate than its parent strain. A lot can be learned about the regulation of fluxes by *S. cerevisiae* if we comprehend this 'enhanced growth rate phenotype' of the *hxk2* deletion strain.

In the third chapter devoted to the physiology of the *hxk2* deletion strain (Chapter 6), consumption of mixed substrates was studied. Due to glucose repression, *S. cerevisiae* will consume glucose preferentially over other carbon sources and will consume mixed carbon sources sequentially. The deletion of the *HXK2* gene enabled co-consumption of sucrose/glucose, galactose/glucose and maltose/glucose. To our surprise this strain even co-consumed glucose and ethanol. Also in this case it would be intriguing to study the expression pattern and comprehend how this strain copes with the combined growth on glucose and ethanol (are gluconeogenetic enzymes expressed, is there futile cycling?).

In the last experimental chapter (chapter 7) the ethanol production in batch cultures of the *hxk2* deletion strain could be reduced further by adding more biotin to the growth medium. Strong up-regulation of biotin-related genes, demonstrated *via* DNA array experiments, led us to this finding. The higher demand for biotin could be explained by an increased demand for biotin to allow the high specific growth rates at high biomass concentrations. These data confirm that the answer to successful manipulation of fluxes resides in controlling regulatory proteins rather than in overexpressing the catalytic enzymes of a pathway [189,217].