Chapter 1
Introduction to Carbon Metabolism in Yeast

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1.1 A Brief History of Yeast Carbon Metabolism

Yeast fermentation of different plant carbohydrate sources, like grape must, is one of the oldest human technologies and its origins date back to the Neolithic period. These fermentations were initially spontaneous and their chemical, physiological, or microbiological background were not understood until the late eighteenth century. The first scientific studies were presented by Antoine Lavoisier in 1789, and he was the first who proposed the chemical changes, conversion of sugar into alcohol and CO_2, occurring during wine fermentation. In 1836–1837 Charles Cagniard-Latour, Friedrich Kützing, and Theodor Schwann independently found that alcoholic fermentation was carried out by living organisms, the sugar fungus. Starting in the late 1850s Louis Pasteur carried out physiological studies; he also clearly demonstrated the role of yeast in alcoholic fermentation, and determined the quantitative differences between aerobic and anaerobic conversion of sugar. Under Pasteur's influence, Emil Christian Hansen isolated the first pure yeast cultures and used them in malt-based fermentation, thereby starting a revolution in brewing industry (reviewed in Barnet 1998, 2003a).

In 1897, Eduard Buchner carried out fermentation by cell-free extracts and opened the way to determine the main biochemical steps. Further studies of yeast and muscle revealed the pathway of glycolysis and demonstrated that under anaerobic conditions pyruvate in yeast was converted into ethanol, whereas muscle

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J. Piškur and C. Compagno (eds.), *Molecular Mechanisms in Yeast Carbon Metabolism*,
DOI: 10.1007/978-3-642-55013-3_1,

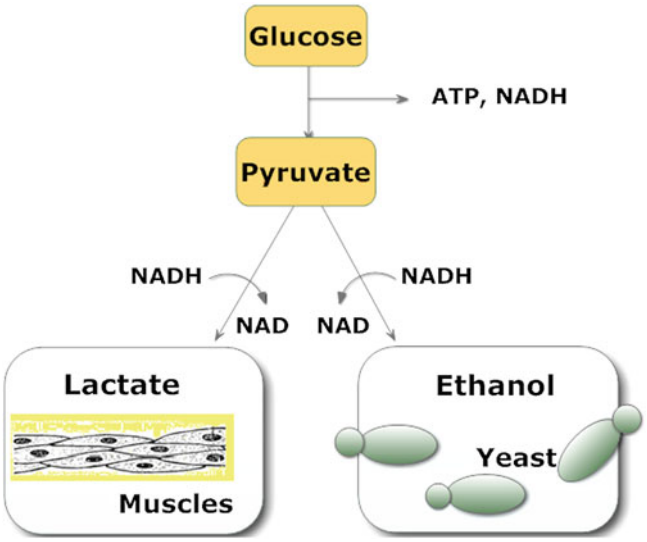

Fig. 1.1 Generation of lactate and ethanol. Sugar is in general degraded to pyruvate and later to CO_2, releasing the stored chemical energy. However, if the availability of oxygen is low, then pyruvate can be reduced either to lactate (in the muscle cell) or to ethanol (in the yeast cell). In this way only little ATP is generated but the NAD/NADH balance is preserved

converted it to lactate (Fig. 1.1). Soon afterward, it became recognized that glycolysis was a universal biochemical pathway found in all organisms and yeast one of the central model organisms to understand this crucial pathway. In the first half of the twentieth century, the discovery of phosphorylated compounds, phosphate bond energy, NAD, and NADH, added important details to the understanding of the energy and redox aspects of the glycolytic and fermentation pathways (reviewed in Barnett 2003b, c).

From the mid-twentieth century, yeast became one of the central model organisms to study the role of genes behind different physiological and biochemical traits. Availability of the first mutants opened a new window to understand the molecular background of alcohol fermentation. For example, in the 1950s and 1960s a lot of focus was on respiration deficient mitochondrial mutants, the so-called *petites* (for review see Piskur 1994).

Over the last few decades, gene sequences have enabled us to study and understand regulatory mechanisms determining the quantitative outcome of sugar conversion into ethanol. In 1996, *Saccharomyces cerevisiae* became the first eukaryote to have its genome fully sequenced (Goffeau et al. 1996). This represented the beginning of the genomics and post-genomic era (for review see Piskur and Langkjaer 2004), which provided new in silico-based approaches and tools, such as comparative genomics, transcriptomics, proteomics, and metabolomics. These global approaches can now provide further insight into the regulatory networks operating at various levels in the yeast carbon metabolism.

1.2 Yeast Biodiversity

In 2011, almost 99,000 fungal species were described, and more recent data from high-throughput sequencing approaches supported an estimate of over 3.5 million species; thus, a majority of fungi still needs to be described (McLaughlin et al. 2009). While a majority of fungi live as multicellular organisms, several lineages have independently adopted a unicellular life mode, the so-called yeast form.

In nature, yeasts are the predominant group of organisms involved in breakdown of simple carbohydrates, especially in the autumn when fruits ripen. Yeasts also include important industrial organisms, pathogens, and popular laboratory organisms that serve as general models to understand the eukaryotic cell. For decades *S. cerevisiae*, baker's yeast, has been one of the best characterized organisms from the genetics, biochemistry, and physiology points of view, and the first eukaryote with a sequenced genome. In analogy with fungi in general, several hundred yeast species have been described so far, but these represent only a small fraction of yeast biodiversity on our planet (Kurtzman et al. 2011).

Within Ascomycota two yeast lineages are well-studied, one is called *Saccharomycetes* (*Hemiascomycetes*) and includes *S. cerevisiae*, and the other is *Schizosaccharomycetes* and includes the fission yeast *Schizosaccharomyces pombe* (Fig. 1.2) The two groups, separated more than 500 million years ago, have independently developed yeast life-forms (Medina et al. 2011). Several yeasts can also be found among *Basidiomycota*.

The *Hemiascomycetes* clade includes several interesting yeast groups, like the pathogenic *Candida* group (including *Candida albicans*), the wine yeasts *Dekkera/Brettanomyces*, the methylotrophic yeasts *Komagataella* (including *Komagataella/Pichia pastoris*, and the *Saccharomycotina* group (Kurtzman et al. 2011).

Many of the industrially interesting yeasts belong to *Saccharomycotina,* and this family covers over 200 million years of the yeast evolutionary history; and includes six post-whole genome duplication (post-WGD) genera: *Saccharomyces*, *Kazachstania*, *Naumovia*, *Nakseomyces*, *Tetrapisispora,* and *Vanderwaltozyma*; and six non-WGD genera: *Zygosaccharomyces*, *Zygotorulaspora*, *Torulaspora*, *Lachancea*, *Kluyveromyces,* and *Eremothecium* (Kurtzman and Robnett 2003; Casaregola et al. 2011). A rough phylogenetic relationship among a few *Hemiascomycetes* yeasts, thoroughly presented in later chapters, is shown in Fig. 1.2.

1.3 Sugar Uptake

Yeast needs a supply of energy for growth and maintenance of the biological order in the cell. This energy comes from the chemical energy stored in food molecules, such as carbohydrates. Before sugars, such as glucose and fructose, can be utilized in the cell, they need to be transported through the plasma membrane. Monohexose uptake in yeast is mainly mediated by hexose transporters (*HXT*). In *S. cerevisiae*

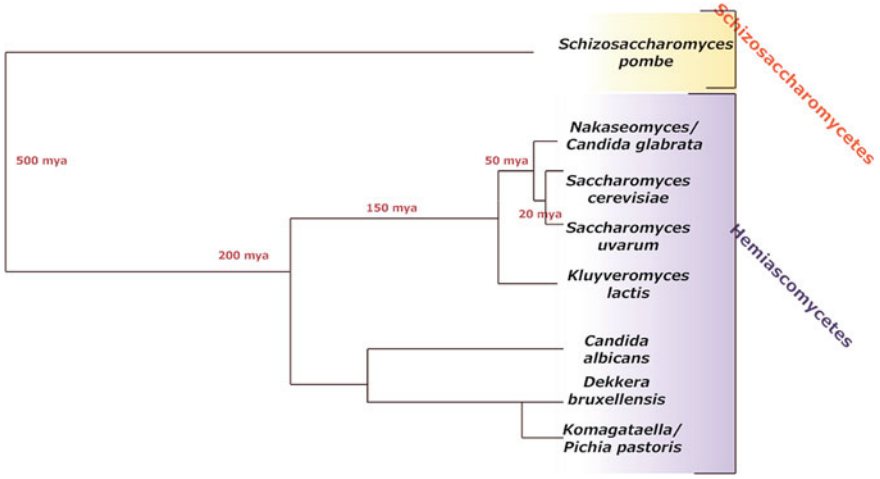

Fig. 1.2 Phylogenetic relationship among some studied yeasts. Note that some of the shown yeast lineages separated from each other many million years ago and have therefore accumulated several molecular and physiological changes regarding their carbon metabolism

all monohexose transport takes place by facilitated diffusion. The *HXT* family in this yeast has 20 members, identified as *HXT1-17*, *GAL2*, *SNF3,* and *RGT2,* and they differ in their affinity toward glucose.

Snf3p and Rgt2p act as sensors of extracellular glucose, in particular, Snf3p senses low glucose levels, and Rgt2p high glucose levels (Özcan and Johnston 1999). In this way *S. cerevisiae* can detect the availability of glucose in the environment and respond by expressing the appropriate transporters. This is due to the combined action of different regulatory mechanisms, including transcriptional regulation of some *HXT* genes and inactivation of Hxt transporters in response to extracellular glucose (Sabina and Johnston 2009). The high-affinity transporters are most useful when glucose is scarce, and the corresponding genes are repressed by high glucose levels. The low-affinity glucose transporter Hxt1 is, on the contrary, expressed when glucose is abundant (Diderich et al. 1999; Kaniak et al. 2004). This controlled expression can then allow *S. cerevisiae* to fine tune sugar uptake in response to the substrate availability.

Also, the uptake of amino acids and nucleotide bases is relatively well-studied in several yeast models. The ability to use different amino acids, purines, and pyrimidines varies among different yeasts (Kurtzman et al. 2011). On the other hand, the uptake of free fatty acids has not yet been fully characterized in yeasts (Casal et al. 2008; see also further chapters).

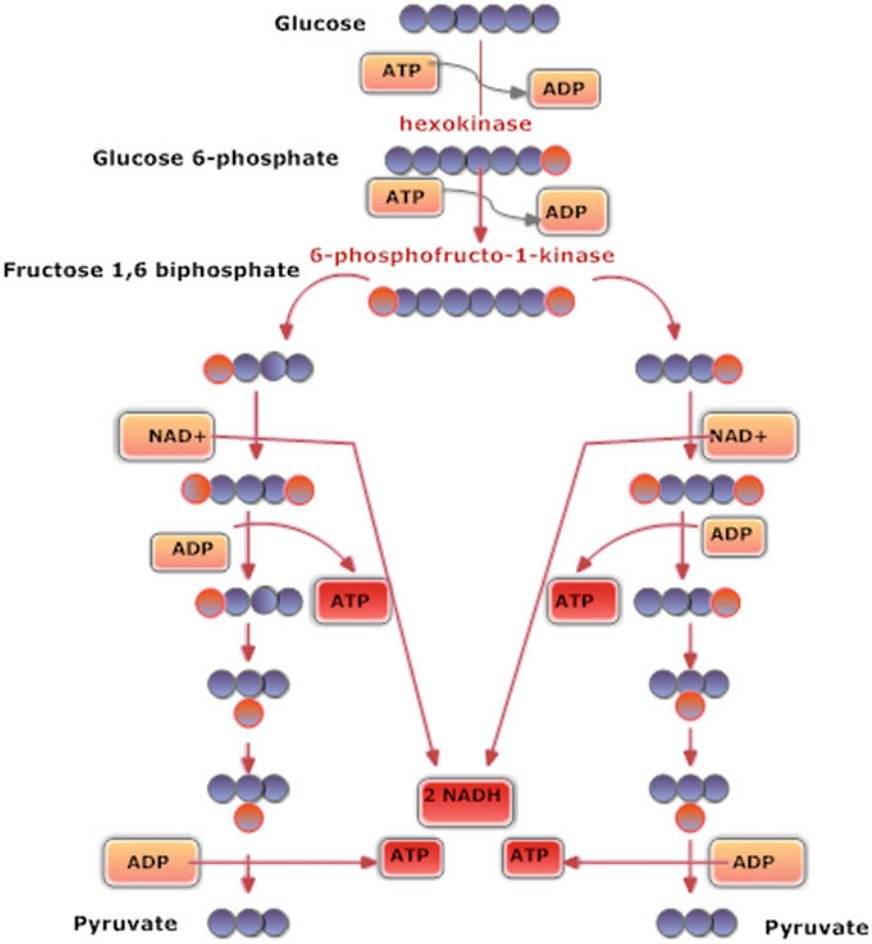

Fig. 1.3 Glycolytic pathway degrades glucose to pyruvate. Glycolysis is a several-step degradation pathway found in many bacteria and almost all eukaryotes. Carbon molecules are shown as *blue circles*, and phosphates as *red circles*. Only the most central intermediates and enzymes are presented. The final products obtained from one molecule of glucose are two molecules of pyruvate, two reduced NADH molecules, and two ATP molecules

1.4 Glycolysis and Fermentation

Sugars, which are preferable yeast food, must be broken down into smaller molecules to become a source of energy and building blocks for the synthesis of other molecules. The major initial process for oxidizing sugars, glycolysis, is a series of ten reactions breaking down a glucose molecule into two molecules of pyruvate. During glycolysis, the cell produces ATP and NADH, without the involvement of molecular oxygen (Fig. 1.3). In the first step, glucose is activated by hydrolysis of

one ATP molecule to generate glucose 6-phosphate. In *S. cerevisiae*, this reaction is catalyzed by three hexokinases (EC 2.7.1.1) encoded by *HXK1*, *HXK2,* and *GLK1*. Hxk2p plays also an important role in the regulation of glucose metabolism, being involved in the mechanism of glucose repression (Klein et al. 1998; Palaez et al. 2010). Later, another ATP molecule is used to generate a central intermediate fructose 1,6-biphosphate, which may play one of the central roles in the regulation of the carbon flow in the cell. The key enzyme catalyzing the irreversible generation of fructose 1,6-biphosphate is 6-phosphofructo-1-kinase (EC 2.7.1.11) and is encoded by *PFK1* and *PFK2*. Studies on the regulation of glycolytic genes indicate that glucose strongly induces the expression of *PFK1* and *PFK2* (Moore et al. 1991). On the other hand, phosphofructokinase activity is inhibited by ATP and citrate and activated by other adenine nucleotides and fructose-2,6-bisphosphate (F2,6P).

Fructose 1,6-biphosphate is converted into two three-carbon products, glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. These two intermediates can be reversibly interconverted. The two three-carbon molecules are oxidized in several steps and electrons removed by NAD resulting in two NADH molecules (from one glucose molecule). The reduced equivalents need to be later re-oxidized in the respiratory chain or during the fermentation pathways to keep the redox balance. Four ATP molecules are also generated during the last glycolytic steps resulting in the final production of two ATP molecules per molecule of glucose. The final glycolysis products are also two molecules of pyruvate. In a majority of organisms, pyruvate is transported into the mitochondria, where it is converted into acetyl CoA and CO_2, and the former later completely oxidized in the presence of molecular O_2 into CO_2 and H_2O. The conversion of pyruvate into acetyl CoA is catalyzed by the pyruvate dehydrogenase complex in the mitochondria. However, in many yeasts, cytoplasmic pyruvate enters into the alcoholic fermentation pathway (Fig. 1.4). Yeasts, depending on conditions, can use sugars by fermentation and/or by respiration (Flores et al. 2000). Since respiration of sugars is energetically more favorable than fermentation, most organisms use fermentation only when respiration is impaired, for example when oxygen availability decreases. However, in several yeast species, like *S. cerevisiae*, the metabolic destiny of pyruvate formed at a high rate is largely switched from respiration to fermentation even when oxygen is abundant. In other words, *S. cerevisiae* may ferment sugars also under aerobic conditions, showing the so-called "Crabtree positive" phenotype (for review see Pronk et al. 1996; Piskur et al. 2006).

The first step in the production of ethanol from pyruvate is the cytosolic decarboxylation to acetaldehyde and CO_2 by the enzyme pyruvate decarboxylase (EC 4.1.1.1). In *S. cerevisiae* three genes encode this enzymatic activity, *PDC1*, *PDC5,* and *PDC6*. *PDC2* encodes a positive regulator of the transcription of *PDC1* and *PDC5* (Hohmann and Cederberg 1990; Hohmann 1993). Acetaldehyde is then reduced to ethanol by the activity of alcohol dehydrogenase (EC 1.1.1.1), leading to the cytosolic re-oxidation of glycolytic NADH. In *S. cerevisiae* five genes encode alcohol dehydrogenases involved in ethanol metabolism (Thomson et al. 2005). Four of these enzymes, Adh1p, Adh3p, Adh4p, and Adh5p, preferentially reduce

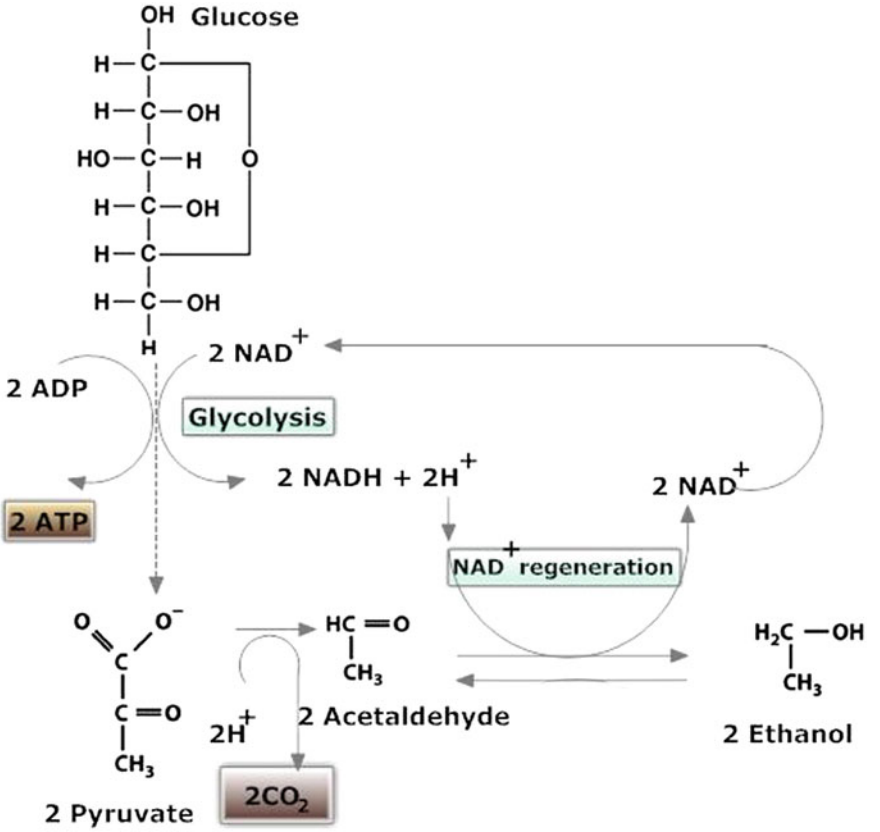

Fig. 1.4 Alcohol fermentation pathway. NADH generated in the glycolytic pathway is re-oxidized to NAD by the help of acetaldehyde reduction to ethanol. The regeneration of NAD thus results in redox balance of the glycolytic and fermentation pathways. The conversion of acetaldehyde into ethanol is reversible and catalyzed by alcohol dehydrogenases

acetaldehyde to ethanol during glucose fermentation, whereas Adh2p preferentially catalyzes the reverse reaction of oxidizing ethanol to acetaldehyde. As described elsewhere in this book, the alcohol fermentation pathway is crucial for production of bio-ethanol and alcoholic beverages.

In addition to this route, pyruvate can also be converted into acetyl CoA at the cytoplasmic level by "pyruvate dehydrogenase bypass." This pathway involves the conversion of acetaldehyde into acetic acid, which is then converted into acetyl CoA. The involved enzymes are an NADP-dependent acetaldehyde dehydrogenase isoform (Ald6p) and an acetyl CoA synthetase (van den Berg and Steensma 1995; Saint-Prix et al. 2004). This bypass route has in *S. cerevisiae* been demonstrated to be essential for growth on glucose, because it is the only source of cytoplasmic acetyl CoA and thus necessary for lipid biosynthesis (van den Berg and Steensma 1995).

1.5 Respiration Part I: Krebs Cycle and Fatty Acid Beta-Oxidation

In eukaryotes, if oxygen is present, the pyruvate produced during glycolysis is transported from cytoplasm into the mitochondria. There, it is decarboxylated and oxidized by a complex of three enzymes, called pyruvate dehydrogenase. In yeast, the decarboxylation reaction is catalyzed by the E1 alpha subunit (EC 1.2.4.1), encoded by the *PDA1* gene. The products are acetyl CoA, NADH, and CO_2. *S. cerevisiae* can also use fatty acids as carbon and energy sources. In this case a remarkable proliferation of peroxisomes is observed, in which fatty acid beta-oxidation occurs (Hiltunen et al. 2003). Fatty acids are broken down by a cycle of reactions, which remove two carbons at a time from their carboxyl end, generating acetyl CoA (Trotter 2001). The main export route of this compound is through the peroxisomal isoform of citrate synthase Cit2p (see also below), and citrate is then able to leave the peroxisomal compartment. Export of acetyl CoA is also accomplished through its conjugation to carnitine by the carnitine acetyl transferase Cat2p, which is localized in both peroxisomes and mitochondria. This pathway is only possible when yeast cells are grown in rich media that contain carnitine, which otherwise cannot be synthesized by the yeast cell. Acetyl CoA is a central intermediate where the sugar and fatty acid degradation pathways meet (Fig. 1.5). The acetyl CoA molecules still store a majority of useful energy and this gets first released in the subsequent degradation cycles.

The citric acid cycle, also known as the tricarboxylic (TCA) acid cycle or the Krebs cycle, accounts for a majority of the total oxidation of carbon compounds in most cells. The Krebs cycle is also an important provider for several substrate molecules, which are crucial in de novo biosynthesis of several amino acids and other essential cellular compounds. Acetyl CoA reacts with oxaloacetate in a reaction catalyzed by citrate synthase (EC 2.3.3.1) to produce citrate. Three genes encoding citrate synthases have been identified in *S. cerevisiae*: *CIT1* and *CIT3* encoding mitochondrial enzymes, and *CIT2* encoding the peroxisomal isoenzyme. In the next step citrate is converted into isocitrate by aconitase (Jia et al. 1997).

The genes corresponding to several of the Krebs cycle and fatty acid catabolism enzymes have been well characterized (Ciriacy 1977; Huynen et al. 1999; Black and DiRusso 2007). *IDH1* and *IDH2* encode the two subunits of the NAD-dependent isocitrate dehydrogenase (EC 1.1.1.41), which transforms isocitrate in 2-oxoglutarate. Another mitochondrial NADP-specific isocitrate dehydrogenase catalyzes the oxidation of isocitrate to 2-oxoglutarate, encoded by *IDP1*, but other isoforms exist; a cytosolic one is encoded by *IDP2* and a peroxisomal one by *IDP3*. The mitochondrial 2-oxoglutarate dehydrogenase complex (EC 1.2.4.2) catalyzes the oxidative decarboxylation of 2-oxoglutarate to form succinyl-CoA. Succinate dehydrogenase (EC 1.3.5.1), composed of four subunits (Sdh1p, Sdh2p, Sdh3p, Sdh4p), couples the oxidation of succinate to the transfer of electrons to ubiquinone as part of the TCA cycle and the mitochondrial respiratory chain. FAD binding to Sdh1p is required for the assembly of the succinate dehydrogenase

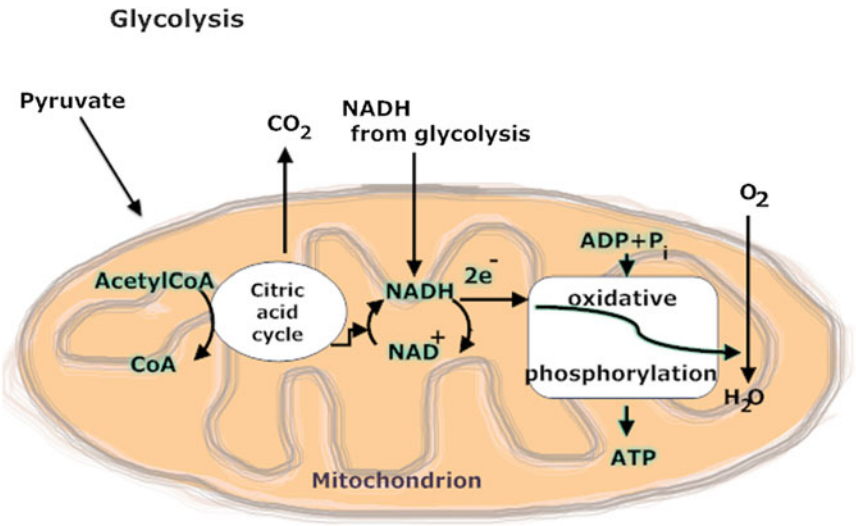

Fig. 1.5 The respiratory part of the sugar catabolism. If oxygen is available as the terminal acceptor of sugar electrons, pyruvate enters mitochondria and is converted into acetyl CoA. During the citric acid cycle CO_2 and reduced equivalents, such as NADH, are generated. NADH is re-oxidized during a cascade of reactions within the respiratory chain, a proton gradient is generated, and oxygen becomes the final acceptor of the electrons and is reduced to water. The proton gradient drives the synthesis of ATP

subunits. The fumarate molecule is then converted by fumarase (EC: 4.2.1.2) into L-malic acid in the TCA cycle; its cytosolic and mitochondrial distribution is determined by the N-terminal targeting sequence, protein conformation, and status of glyoxylate shunt. Mitochondrial malate dehydrogenase (EC 1.1.1.37), encoded by *MDH1* gene, catalyzes interconversion of malate and oxaloacetate. The cytoplasmic form (encoded by *MDH2*) and the peroxisomal one (encoded by *MDH3*) catalyze interconversion of malate and oxaloacetate in the glyoxylate cycle, during growth on two-carbon compounds as well as on fatty acids (described below).

1.6 Respiration Part II: Respiratory Chain and ATP Synthase

The major end products of acetyl CoA degradation through TCA cycle are CO_2 and high-energy electrons stored in NADH. During the respiration part, the NADH electrons are passed to a membrane-bound electron-transport cascade. The respiratory chain consists of several complexes: NADH dehydrogenase complex, cytochrome b-c_1 complex, and cytochrome oxidase complex (Fig. 1.5). In combination with oxygen, which is the terminal acceptor of electrons, proton gradient and H_2O are generated. It is interesting to point out that the genes coding for the

respiratory chain elements can be found in the nuclear as well as in the mitochondrial genome. One of the first studied classes of yeast mutants, called *petites*, were respiratory deficient strains with mitochondrial DNA, which had lost the coding ability for the respiratory chain and/or ATP synthase (reviewed in Piskur 1994). The cytochrome bc1 complex (also known as ubiquinol:cytochrome c oxidoreductase, ubiquinol:ferricytochrome c oxidoreductase, and respiratory complex III) (EC 1.10.2.2) is a highly conserved enzyme of the mitochondrial respiratory chain (Smith et al. 2012). In *S. cerevisiae* it consists of three catalytic subunits, Cobp, Rip1p, and Cyt1p, plus seven additional subunits: Cor1p, Qcr2p, Qcr6p, Qcr7p, Qcr8p, Qcr9p, and Qcr10p.

The nuclear *CYC1* and *CYC7* genes and the corresponding products, iso-1-cytochrome c and iso-2-cytochrome c are among the most thoroughly studied gene-protein systems (Sherman 1990) and pioneered the development of yeast molecular genetics. The cytochrome c oxidase complex (EC 1.9.3.1) catalyzes the terminal step in the electron transport chain involved in cellular respiration (Soto et al. 2012). This multisubunit enzyme of the mitochondrial inner membrane, also known as Complex IV, is composed of three core subunits encoded by the mitochondrial genome (Cox1p, Cox2p, and Cox3p) and eight additional subunits encoded by nuclear genes (Cox4p, Cox5Ap or Cox5Bp, Cox6p, Cox7p, Cox8p, Cox9p, Cox12p, and Cox13p).

The electron transport, through the established proton gradient, drives the synthesis of the majority of ATP (Fig. 1.5). The generation of ATP is catalyzed by F_1F_0 ATP synthase, which is a large, evolutionarily conserved enzyme complex required for ATP synthesis (EC 3.6.3.14) (Stuart 2008).

The efficiency of proton pumping by respiratory chain and ATP synthesis is represented by the P/O ratio (ATP formed per electron pair transferred to oxygen). Several independent yeast lineages, including *S. cerevisiae*, do not have Complex I. As a result, its effective P/O ratio is low, close to 1, and the complete glucose oxidation probably only yields 16 ATP (Bakker et al. 2001). In contrast, in mammals the complete oxidation of a glucose molecule can in theory produce about 30 molecules of ATP. On the other hand, during the glycolytic and fermentation pathways only 2 ATP molecules can be produced. In some yeast species alternative oxidases that transfer electrons from cytochrome c to molecular oxygen without proton translocation have been described (Veiga et al. 2003).

1.7 Glycolysis Reversed: Gluconeogenesis and Glyoxylate Cycle

Metabolism consists of catabolic and anabolic pathways. In the previous sections we mainly focused on the catabolic or degradation aspects. Gluconeogenesis is the process whereby glucose and other sugars are synthesized from other precursors, enabling yeast cells to grow on ethanol, glycerol, or peptone (amino acids).

In short, this pathway provides hexose building blocks when the yeast food consists of only C2 and C3 compounds (non-fermentable carbon sources). Reactions of gluconeogenesis mediate the conversion of pyruvate into glucose, in a sense the opposite of glycolysis. Overall, the gluconeogenic reactions convert two molecules of pyruvate into a molecule of glucose, with the expenditure of six high-energy phosphate bonds, four from ATP and two from GTP. While the two pathways, glycolysis and gluconeogenesis, have several reactions in common, they are not the exact reverse of each other. As the glycolytic enzymes 6-phosphofructo-1-kinase (EC 2.7.1.11) and pyruvate kinase (EC 2.7.1.40) only function in the forward direction, the gluconeogenesis pathway replaces those steps with the enzyme phosphoenolpyruvate carboxykinase (EC 4.1.1.49, encoded by *PCK1*), generating phosphoenolpyruvate from oxaloacetate, and with the enzyme fructose-1,6-bisphosphatase (EC 3.1.3.11, encoded by *FBP1*) to generate fructose-6-phosphate.

Intermediates from the TCA cycle are removed for biosynthesis, and the cycle needs to be replenished. The enzyme pyruvate carboxylase (EC 6.4.1.1, encoded by *PYC1* and *PYC2*) generates oxaloacetate from pyruvate. The glyoxylate cycle comprises many of the same reactions as the Krebs cycle, but intermediates and enzymes are located in the yeast peroxisome and cytoplasm, and decarboxylation enzyme activities are missing. Thus, this cycle allows cells to utilize simple carbon compounds as an energy and carbon source when glucose is absent. Two-carbon substrates, such as acetate or ethanol, can enter as acetyl CoA and are converted into four-carbon compounds and later to other essential compounds (Strijbis and Distel 2010). This cycle can also be a provider of NADH for de novo biosynthetic reactions and helps to balance the redox potential in the cell. Enzymes activities specific for this cycle are isocitrate lyase (EC 4.1.3.1 encoded by *ICL1*) and malate synthase (EC: 2.3.3.9 encoded by *MLS1*).

1.8 Aerobic and Anaerobic Yeasts

The availability of oxygen varies among different niches. One of the main problems an organism faces under anaerobic conditions is the lack of the final electron acceptor in the respiratory chain. This reduces or completely eliminates the activity of Krebs cycle, respiratory chain, and mitochondrial ATP synthases. As a response to hypoxic and anaerobic conditions, organisms have developed several processes to optimize the utilization of oxygen and even reduce the dependence on the presence of oxygen. According to the dependence on oxygen during the life cycle, yeasts are classified as: (i) obligate aerobes displaying exclusively respiratory metabolism, (ii) facultative fermentatives (or facultative anaerobes), displaying both respiratory and fermentative metabolism, and (iii) obligate fermentatives (or obligate anaerobes) (Merico et al. 2007).

The ability of yeasts to grow under oxygen-limited conditions seems to be strictly dependent on the ability to perform alcoholic fermentation. In other words, enough ATP should be generated during glycolysis to support the yeast growth,

and NADH generated during glycolysis gets re-oxidized. The yeast also needs to develop resistance to larger quantities of the final fermentation product, ethanol, as well as any other fermentation products. Apart from the energy and NADH/NAD redox problems, under anaerobic conditions, yeast must also find a way to run various reactions independent of the respiratory chain and a normal Krebs cycle. In other words, substrates (intermediates) for de novo reactions, for example for the amino acid pathways (synthesis), need to originate from a modified metabolic network. One of the alternative providers is the glyoxylate cycle. However, as detailed elsewhere in this book, under anaerobic growth, some mitochondrial Krebs cycle enzymes must be active, at least to generate 2-oxoglutarate.

Anaerobically growing *S. cerevisiae* cells contain only a few large, branched mitochondria (Visser et al. 1990). Under these conditions the mitochondria do not play a role in free-energy metabolism. However, important assimilatory reactions still take place in the mitochondria, generating NADH, which then needs to be re-oxidized (Nissen et al. 1997). During anaerobic growth in *S. cerevisiae* the Krebs cycle operates as two branches, but there is no flux from 2-oxoglutarate to fumarate (Nissen et al. 1997). Anaplerotic pathways and the glyoxylate cycle provide intermediates for anabolic reactions. Glycerol production is the primary redox sink for the re-oxidation of NADH, under anaerobic conditions (van Dijken and Scheffers 1986). The mitochondrial form of alcohol dehydrogenase Adh3p is essential to couple the reoxidation of mitochondrial NADH and glycerol formation (Bakker et al. 2000).

On the other hand, some yeast compounds, like unsaturated fatty acids and sterols, cannot be synthesized in the cell under anaerobiosis and must originate from the medium or from previous aerobic growth. Also, the ability to translocate ATP generated in the cytoplasm into the mitochondria, for biosynthetic purposes, should be well developed to survive in the absence of oxygen. In *S. cerevisiae*, the presence of the mitochondrial ATP transporters, encoded by *AAC2* and *AAC3*, is essential for growth under anaerobic conditions (Sabova et al. 1993; Betina et al. 1995).

1.9 Physiological Aspects: Crabtree Effect

One of the most prominent features of the baker's yeast *S. cerevisiae* is its ability to rapidly convert sugars into ethanol and carbon dioxide at both anaerobic and aerobic conditions. When oxygen is absent, acetaldehyde is the final electron acceptor and gets converted into ethanol under purely fermentative growth. Under aerobic conditions, respiration is possible with oxygen as the final electron acceptor, but *S. cerevisiae* still exhibits alcoholic fermentation until the sugar/glucose reaches a low level (Fig. 1.6). This phenomenon is called the Crabtree effect (De Deken 1966) and the yeasts expressing this trait called Crabtree-positive yeasts. In contrast, "Crabtree-negative" yeasts lack fermentative products, and under aerobic conditions, biomass and carbon dioxide are the sole products.

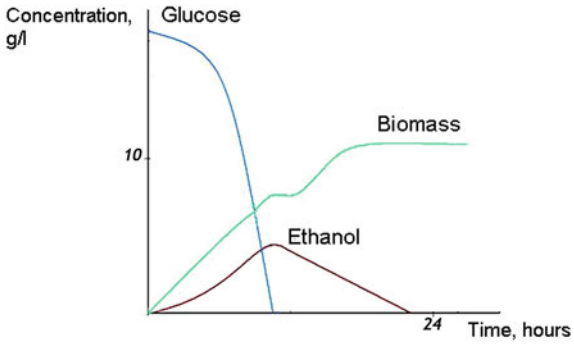

Fig. 1.6 Batch culture of a Crabtree-positive yeast. The yeast was grown under aerobic conditions in a defined minimal medium and the disappearance of glucose and the appearance of biomass and ethanol were followed. When glucose disappeared, a characteristic diauxic growth occurs, due to the utilization of ethanol, previously generated from glucose, as the main carbon source. This represents a switch between fermentative and respiratory metabolism

However, it is possible to obtain pure respiratory utilization of glucose by *S. cerevisiae* under aerobic conditions if the glucose concentration is kept very low in the medium, e.g., by using a glucose-limited continuous culture operating below a certain strain-specific threshold value (called "critical" dilution rate) or by using fed-batch cultivations (Postma et al. 1989). Briefly, glucose is sensed by the yeast cell, and this signal is transmitted further to diminish the respiratory activities (see further chapters). This glucose repression phenomenon involves different signal transduction pathways activated by extracellular and intracellular levels of glucose and related metabolites and/or their fluxes through the key glycolytic enzymes (reviewed in Klein et al. 1998; Johnston 1999; Westergaard et al. 2007). In other words, the complexity of glucose repression regulatory networks is still far from being completely understood. Some of the regulatory activities operate at the transcriptional regulation level and some may operate directly on the involved enzymes and their regulators.

There does not seem to be a clear consensus about the definition of the Crabtree effect, and different physiological and molecular approaches have been used as the background for the current definitions (von Meyenburg 1969; Barford and Hall 1981; Kappeli 1986; Alexander and Jeffries 1990). We define the long-term Crabtree effect as aerobic alcoholic fermentation under steady-state conditions at high growth rates. When *S. cerevisiae* is cultivated in a glucose-limited chemostat, the long-term effect appears when the dilution rate (or in other words: the glucose uptake rate) exceeds the strain-specific threshold value. The same effect is observed also when yeast cells are cultivated in glucose-based conditions, e.g., batch cultivations. The molecular background for the long-term Crabtree effect seems to be a limited respiratory capacity due to the repression of the corresponding respiration associated genes (Postma et al. 1989). On the other hand, we define the short-term Crabtree effect as the immediate appearance of aerobic alcoholic fermentation upon

addition of excess sugar to sugar-limited and respiratory cultures. This effect has also been explained as an overflow in the sugar metabolism and could be associated directly with the biochemical properties of the respiration-associated enzymes and their regulators (Petrik et al. 1983; Postma et al. 1989; Pronk et al. 1996). In addition, it could depend on immediate repression of some key genes involved in respiration. However, it is still unclear if the regulatory molecular mechanisms operating during the long-term and short-term Crabtree effect are indeed different from each other. A very interesting aspect is the evolutionary background for the development of these regulatory mechanisms (Piskur et al. 2006).

The oxygen availability during sugar metabolism can also determine other kinds of effects. The Pasteur effect has been defined as the inhibition of fermentative metabolism by oxygen, but in *S. cerevisiae* this phenomenon is observable only at low glycolytic fluxes (Pronk et al. 1996). In some yeast species the absence of oxygen impairs the utilization of particular disaccharides, although one or both of the monosaccharide components can be used anaerobically by fermentation, the so-called "Kluyver effect" (Fukuhara 2003). This characteristic seems to be determined mainly by the activity of sugar carriers (Goffrini et al. 2002). The inhibition of fermentation of glucose as well as of other sugars in the absence of oxygen has been described as the Custer effect. This effect has been found in some *Brettanomyces/Dekkera* and *Candida* species, and proposed to be due to a redox imbalance (van Dijken and Scheffers 1986), as confirmed in a recent study (Galafassi et al. 2013).

1.10 Regulatory Aspects

In *S. cerevisiae*, the main elements of the glucose sensing systems have been studied from many years, and apparently the main involved elements have been characterized (Fig. 1.7). Snf3p and Rgt2p have been shown to likely act as receptors that sense external glucose concentration, Snf3p for low and Rgt2p for high levels of glucose (Özcan and Johnoston 1999). The signal is then transduced to Rgt1p, a transcription factor for glucose-regulated genes. The signal that causes the repressing capacity of Rgt1p is lost, allowing derepression of the appropriate *HXT* genes and enabling glucose transport. Another sugar signaling mechanism has been described, the Gpr1-Gpa2 system, which is linked to the Ras/cAMP-PKA cascade. This system mediates many effects produced by glucose, as stimulating glycolysis, inhibiting gluconeogenesis, regulating carbohydrate metabolism, as well as cell cycle progression, stress response, and ribosomal biogenesis (Verstrepen et al. 2004). Transcription activators Rap1/Gcr1/Gcr2 and Sfp1 are known to stimulate the transcription of glycolytic and ribosomal protein genes in response to the glucose presence (Shore 1994; Clifton and Fraenkel 1981; Marion et al. 2004).

The presence of glucose is also known to trigger the repression of hundreds of genes. The key elements are: (i) transcription repressors Mig1, Mig2, and Mig3; (ii) protein kinase Snf1; and (iii) protein phosphatase Glc7-Reg1. A drop in level

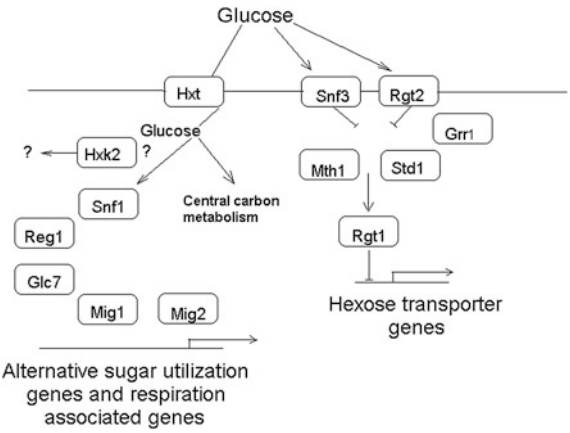

Fig. 1.7 Regulatory aspects of glucose utilization. Some of the main players in yeast glucose sensing and signal transduction are shown. Some of these elements can act as activators or repressors of the terminal genes involved in glucose and other sugars uptake and metabolism

of glucose activates the Snf1 kinase activity, causing phosphorylation of Mig1p, and promotes the Mig1p to leave the nucleus. This results in de-repression of glucose-repressed genes. The sensor of this pathway is not yet known, but it is believed to involve Hxk2p, which also acts in the nucleus as a regulator of the transcription of several Mig-regulated genes (through interaction with Mig1 and Snf1 protein kinase). Under high glucose conditions, in fact, it has been found that nuclear Hxk2p stabilises the repressor complex blocking the phosphorylation of Mig1 by Snf1 kinase (Ahuatzi et al. 2007). Recent developments have provided two important observations: the cross-talk between the different pathways involved in carbon source metabolism and the induction/repression duality of some involved transcription factors, like Mig1, Rgt1, and others (Westergaard et al. 2007; Santangelo 2006; see also further chapters).

An important regulatory system is the Hap2/3/4/5p complex, which activates transcription of genes encoding respiratory chain components and enzymes of the TCA cycle in the absence of easily fermentable carbon sources such as glucose (Schuller 2003). The utilization of non-fermentable substrates requires also the induction/derepression of gluconeogenesis and glyoxylate enzymes, which occurs by carbon source-responsive element (CSRE) binding factor Cat8p (Schuller 2003). *HAP4* and *CAT8* are both glucose repressed genes.

The availability of oxygen is another environmental factor that widely affects cell metabolism. Functional analyses indicate that the transcription factors Rox1p and Upc2p predominate in the regulation of carbon metabolism, lipid metabolism, and cell wall maintenance (Kwast et al. 2002; see also further chapters).

1.11 Evolution of Ethanol Fermentation

A majority of ascomycotic fungi under aerobic conditions convert sugar-based substrates into CO_2. However, at least three groups, including budding and fission yeasts, have apparently independently evolved the metabolic ability to produce

ethanol in the presence of oxygen and excess of glucose (reviewed in Rozpedowska et al. 2011; Rhind et al. 2011). This metabolic invention (Crabtree effect), represents in nature a possible tool to outcompete other microbes. For example when fruits ripen, microbial communities start a fierce competition for the freely available sugars. Yeasts from the *Saccharomyces* clade, which are good ethanol producers and also ethanol-resistant and facultative anaerobes, soon become the predominant microbes in these niches. Both groups of ethanol-producing budding yeast, including *S. cerevisiae* and *Dekkera bruxellensis*, can also efficiently catabolize ethanol, and therefore their corresponding life style has been named as "make-accumulate-consume (ethanol)" strategy (Thomson et al. 2005; Piskur et al. 2006; Rozpedowska et al. 2011). On the other hand, the third Crabtree positive group, including the fission yeast *Sch. pombe*, only poorly metabolizes ethanol (Fig. 1.2).

The onset of yeast genomics (Goffeau et al. 1996) has provided a tool to reconstruct several molecular events, which have reshaped the budding yeasts during their evolutionary history (reviewed in Dujon 2010). Several molecular events have left a clear fingerprint in the modern genomes, while the origin of more complex traits, like the Crabtree effect, is often not easy to determine using only a genome analysis approach. Until recently, very few yeast species have been studied for their carbon metabolism (Merico et al. 2007). In a recent work, over 40 different *Saccharomycetales* yeasts have been studied for the presence of long-term Crabtree effect and it has been found that this effect originated after the split of the *Saccharomyces-Lachancea* and *Kluyveromyces-Eremothecium* lineages, approximately 125 million years ago, prior to the whole genome duplication (WGD) event, and after the loss of the respiratory complex I (Hagman et al. 2013). The origin of modern plants with fruits, more than 125 million years ago, brought to microbial communities a new larger and increasingly abundant source of food based on simple sugars. On the other hand, ancient yeasts could hardly produce the same amount of new biomass as bacteria during the same time interval, and could therefore be outcompeted. We speculate that slower growth rate could in principle be counteracted by production of compounds that could inhibit the growth rate of bacteria, like ethanol and acetate. However, the initial molecular mechanisms that promoted the evolution of the new "lifestyle" and rewiring of the carbon metabolism are so far not identified.

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