SYSTEMS BIOLOGY OF OVERFLOW METABOLISM

by

GOUTHAM N. VEMURI

(Under the Direction of Mark A. Eiteman)

ABSTRACT

The primary goal of this research is to elucidate the regulation of overflow metabolism in *Escherichia coli* and *Saccharomyces cerevisiae*. Insufficient capacity of the respiratory pathways to oxidize NADH is hypothesized to be the cause of this phenomenon. Therefore, I increased NADH oxidation in these two organisms and studied the response from a systems biology perspective. I cloned NADH oxidase in *E. coli* as well as *S. cerevisiae* and observed a reduction in the overflow of acetate and glycerol, respectively, at the cost of biomass synthesis. I also introduced the cyanide-resistant alternate oxidation pathway in *S. cerevisiae*, which decreased ethanol production. Transcription profiling revealed interesting regulatory mechanisms, based on which I proposed new models for the control of overflow metabolism in these organisms. I believe that this research, spanning over three Universities (University of Georgia, University of Minnesota and Denmark Technical University), has laid the foundation for future redox-mediated metabolic engineering work in *E. coli* and *S. cerevisiae*.

INDEX WORDS: Redox metabolism, *Escherichia coli, Saccharomyces cerevisiae*, Overflow metabolism, NADH oxidase, Alternate Oxidase, Metabolic engineering, Systems biology:

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DEDICATION

To my family

Preface

We've called the genome the blueprint, the Holy Grail, all sorts of things. It's a parts list. If I gave you the parts list for the Boeing 777 and it has 100,000 parts, I don't think you could screw it back together and you certainly wouldn't understand why it flew.

Eric Lander, Millennium Evening at the White House

I write the preface with the lurking suspicion that most readers ignore this part of the thesis. Nevertheless, I write it to share with the interested readers the inspiration behind the research. Every PhD is a unique story. I completed my MS and was contemplating between joining the workforce in the industry and continuing further in my education. Taking up a job in a high profile industry that would help me immigrate to the United States after an MS degree would have been "normal" trend for a typical engineering student from India. Those that enroll in a PhD program end up doing so either because they could not find a suitable job or a job at all. It is only a handful of students who desire to work on a PhD with a passionate desire to enquire into the nature of things. This endeavor comes with a price – sacrificing the salary they would have earned, had they worked in an industry, sacrificing quality time with loved ones and living a life saturated with uncertainties. However, the end result is truly rewarding and is a source of great personal pride. These have been my experiences and I believe most of those with a PhD, if 60

not all, can relate to similar ones. My decision to embark on a PhD journey was neither an

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impulsive one nor compelled by the lack of employment opportunity. On the contrary, it was the consequence of an eminently rational decision process.

In many ways, this thesis represents my transition from working alone on biological problems to collaborating with scientists possessing complementary skills. In other words, it reflects my transformation from a traditional biochemical engineer to a systems biologist. During the course of my doctoral research, I learnt to view cells as biological systems with various components that function synergistically to impart a characteristic phenotype. The complexity of biological systems, even single-celled microorganisms, requires integration of information from different cellular components to appreciate their functioning as a unified system. Working with people from diverse scientific backgrounds and research philosophies, with different cultures and ideas has given me a rich experience, and to successfully complete projects under different environments proved to be task more daunting that I ever imagined.

This PhD thesis documents the results from my research as a doctoral candidate at the Driftmier Engineering Center, University of Georgia in the period of January, 2001 to December 2005. A majority of the results described here transpired from the work I did at the University of Minnesota and Denmark Technical University. I spent the first six months as a doctoral candidate at Shire Biologicals, Northboro, MA working on optimizing the fermentation processes for producing recombinant vaccines. The diverse work environments together with the various aspects of research questions that were addressed make this work very unique and wholesome. The thesis is presented as a compendium of independent manuscripts that are published or soon will be published in scientific journals, each telling a different story. These stories have overflow metabolism as a common thread. Each manuscript is presented as a separate chapter in the thesis and it is not necessary to read the previous chapters of the thesis to understand subsequent chapters. The electronic version of the thesis is equipped with hyperlinks to the appropriate figure, table, section, etc in the hope that it will serve as a good reference for further studies on understanding and developing redox-based metabolic engineering strategies. Moreover, all the transcription data is provided as web-based interactive supplement at http://www.engr.uga.edu/research/cmbe/goutham.

Goutham Vemuri University of Georgia, Athens; GA Lyngby, Denmark May 2007

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It is not out of convention that I include this section, but rather with true and heart-felt gratitude to all the people mentioned below, without whose contribution my doctoral research would not have materialized into its final form.

First and foremost, I thank Mark Eiteman for his mentorship, guidance and support that saw me overcome technical, bureaucratic and personal hurdles during my PhD journey. His constant encouragement inspired confidence in me to proceed with my PhD. I hope that the excellent relationship I have with him continues well beyond my PhD and solidifies further. I also thank Prof. Brahm Verma for his constant moral support during the troubled times that helped me proceed with the work. The discussions I had with him helped me identify the problem and find the most logical solutions. The assistance of Dr. William Kisaalita in technical and bureaucratic problems is very much appreciated. Often, his critical comments provided the much required lubrication for the pistons in my brain to fire. I thank Prof. Claiborne Glover III for introducing me to eukaryotic biology and providing me feedback on several technical issues related to my work with yeast. I also thank Prof. Jaxk Reeves for help with the analysis of the huge amounts of data and Dr. James Kastner for his useful discussions on various aspects of research. I thank Sarah Lee for her friendship and support during my entire stay at the University of Georgia.

I thank Dr. Arkady Khodursky for inviting me to his lab to work on microarrays, where I learned various aspects of studying genome transcription in *E. coli*. I acknowledge the fruitful

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I am very thankful to my entire family for their constant encouragement and support, to whom I dedicate this thesis.

I am very grateful to the University of Georgia Research Foundation and U.S. Department of Energy Biobased Industrial Fellowship for funding my doctoral research and travel to various conferences.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

No question is so difficult to answer as that to which the answer is obvious.

- George Bernard Shaw

Introduction

Biotechnology has emerged to be one of the most important developments in the last century and promises to offer solutions to many challenges faced by the chemical and pharmaceutical industry. The wide spectrum of industries implementing this new technology prompted color-coding of biotechnology applications. For example, medical and pharmaceutical biotechnology was coded red, agricultural and plant biotechnology was coded green and marine biotechnology was coded blue. White biotechnology is an emerging field that specifically caters to the needs of the chemical and environmental industry (Frazzetto, 2003). It relies largely on using living cells like yeast and bacteria as cell factories for sustainable production of biochemicals, biomaterials and biofuels from renewable resources. A recent study conducted by McKinsey and Co. predicts immense growth potential for white biotechnology in the future (Internet Reference 1), with some of the large chemical companies such as BASF and DSM already replacing their chemical processes with cleaner, more efficient bioprocesses.

An important component in developing microbial cell factories for an economically viable, efficient bioprocess is to optimize metabolic networks. Since naturally occurring microorganisms are not adapted for bioprocesses, it is necessary to engineer their genetic architecture to achieve a specific phenotype. Rational alteration of the metabolism to meet an objective is called metabolic engineering and conventionally, the first step in most metabolic engineering applications is the identification of the "rate-limiting" step(s) and overexpressing the gene(s) that affect these steps or inactivating the inefficient pathways that contribute to byproduct formation. While this strategy enjoyed moderate success, more complex aspects of metabolism such as increasing the pH tolerance or expanding the range of consumable substrates could not be achieved. This limitation is because the cells have evolved a complex network of regulatory mechanisms that counteract many mutations by employing alternative pathways for continued robust performance. Therefore, the focus of metabolic engineering is shifting towards engineering regulatory mechanisms.

The recent advances in genome sequencing, proteomics and transcriptomics and metabolite profiling have not translated into success stories in metabolic engineering due to the lack of understanding of regulation, or how the different cellular components interact with each other to produce the phenotype (Vemuri and Aristidou, 2005). We are still far from understanding regulatory phenomena from a global perspective. The high-throughput techniques recently developed have the potential to disclose extremely useful information, but they provide a snapshot from only one stage of transfer of information from gene to function (Figure 1.1) while possibly missing the cause and effect relationships from other stages. A holistic approach of integrating information from genes, proteins and metabolites for a comprehensive understanding of systemic functions is called systems biology, and this approach holds the promise to provide important information about new regulatory mechanisms that will expedite the progress of white biotechnology. Currently, the mathematical models that integrate information lack in either specificity or sensitivity due to a limitation in the biological knowledge available and the mathematical rules to bring the data together. The focus of my doctoral research is on integrating information from the different levels of hierarchy in shown in Figure 1.1, as applied to the specific problem of understanding redox-mediated regulation of metabolism in prokaryotes and eukaryotes. Integration of high throughput data, defined as systems biology,

holds the promise to reveal new regulatory mechanisms that cannot be discovered by conventional reductionist approaches.

Figure 0-1

Organization of the various -omes in a hierarchical fashion. The comprehensive DNA sequence in a cell is the genome, which consists of coding regions, shown as gray bars. The coding process begins with the expression of DNA to the respective mRNA species, which consists of the transcriptome. The transcription process is dictated by several factors, including the interaction of proteins and metabolites with DNA and the presence/absence of the required cellular machinery. The mRNA species are translated to form proteins (proteome). As shown in the figure, there may not exist a one-to-one correspondence between proteins and genes. The various interactions between proteins, DNA, and metabolites, called the interactome, are the key determinants of cellular processes. The solid arrows represent the flow of biological information, while the dashed lines show possible interactions between various cellular components. Proteins are the functional entities that carry out the actual metabolic process by interconverting metabolites (metabolome). Any observed phenotype such as growth and product formation is the net result of all these cellular events. Therefore, capturing information at just one stage of the process (transcription, translation, etc.) will not reveal the cause and effect relationships between cellular components. It is necessary for the metabolic engineer to understand these relationships in order to accurately design and control biological systems. Figure from Vemuri and Aristidou, 2005.



Carbohydrates, particularly glucose, are the most common carbon and energy sources for cells. Most prokaryotic and eukaryotic cells have evolved to possess an extremely efficient glucose consumption mechanism by means of glycolysis. Carbon from glycolysis subsequently enters the TCA cycle, which provides precursors for biomass generation. As the glycolytic flux increases, the carbon flux in the TCA cycle also increases correspondingly. However, beyond a certain value of glycolytic flux the TCA cycle flux saturates, resulting in an overflow of carbon in the glycolysis. This excess carbon flows into reduced end products such as lactate (in mammalian cells), acetate (in *Escherichia coli*) or ethanol and glycerol (in *Saccharomyces cerevisiae*). The production of these typical anaerobic products under fully aerobic conditions by facultative anaerobes is known as overflow metabolism.

Under fully oxidative conditions glucose is converted into pyruvate and CO₂, respectively, and generate reducing equivalents in the form of NADH and FADH₂, as summarized in the following equations.

$$Glu \cos e + 2ADP + 2NAD + 2Pi \rightarrow 2 pyruvate + 2ATP + 2NADH$$
$$Pyruvate + GDP + FAD + 4NAD \rightarrow 3CO_2 + GTP + FADH_2 + 4NADH$$

These reducing equivalents have to be reoxidized in order for the reactions to proceed and for continued consumption of glucose for biomass and energy generation. The oxidation of NADH and FADH₂ occurs in the respiratory chain, and is coupled with energy generation in the form of ATP by oxidative phosphorylation. A simplified schematic of respiration coupled with ATP generation is shown in Figure 1.2.

Figure 0-2

A generalized schematic depicting the transfer of electrons (fine lines) from NADH in a sequential process ultimately to oxygen, resulting in the generation of NAD to fuel sugar dissimilation and concomitant synthesis of ATP. The thick lines illustrate proton translocation. The figure illustrates various components involved in the process, as well as the translocation of



protons by these complexes.

In prokaryotes, glycolysis and the TCA cycle occur in the cytoplasm and the NADH that is generated from these reactions can easily enter the respiratory chain. In eukaryotes glycolysis occurs in the cytosol while the TCA cycle and respiration are localized in mitochondria. Furthermore, since the mitochondrial membrane is not permeable to the reduced cofactors, cytosolic NADH and mitochondrial NADH must be oxidized in the compartments in which they are generated using dedicated mechanisms. Consequently, cytosolic NADH cannot directly participate in oxidative phosphorylation and ATP generation. It is widely known that the ratio of NADH to NAD, also known as the redox ratio, dictates several metabolic characteristics, and is a key parameter in determining the efficiency of energy generation and product formation (Iuchi and Lin, 1993; Bakker et al., 2001). The limited capacity of the cells in oxidizing NADH is believed to be the cause for overflow metabolism.

The widespread role of redox in regulating several cellular functions involving transcriptional and post-transcriptional processes makes it an ideal topic of interest for systems biology approach. The fundamental difference in the cellular organization of metabolism and respiration between prokaryotes and eukaryotes offers an excellent opportunity to compare and contrast the redox-dependant regulatory mechanisms in these two systems. In this work *Escherichia coli* and *Saccharomyces cerevisiae* were used as the model prokaryote and eukaryote, respectively, to study NADH reoxidation and its subsequent influence on metabolism. In addition to being the microorganisms of choice for use as cell factories owing to their well established genetic manipulation techniques and physiology, *E. coli* and *S. cerevisiae* are both facultative anaerobes.

Since the redox ratio ultimately determines the mode of metabolism in each of these organisms, they are obvious choices for this research.

In *E. coli*, the oxidation of NADH generated from glycolysis and the TCA cycle occurs sequentially in a stepwise manner involving the respiratory chain, also known as the electron transport chain (ETC), as shown in Figure 1.2. Electrons from NADH are transported to ubiquinone, and ultimately to oxygen by means of cytochrome oxidases, while concomitantly generating a proton motive force (PMF). Therefore, the redox potentials of the various components in the ETC vary between that of NAD ($E_0 = -320$ mV) and O_2 ($E_0 = +820$ mV). *E. coli* generates PMF using the cytoplasmic membrane-bound NADH dehydrogenase I (encoded by the *nuoA-N* genes). This route dominates NADH oxidation in *E. coli*, but surplus NADH activates another route (Steuber, 2001) which is less efficient and non-PMF generating: NADH dehydrogenase II (encoded by *ndh* gene). How *E. coli* regulates the degree of functioning of these two routes for NADH oxidation is still unclear. The F₀F₁-ATPase uses the PMF to generate ATP and the oxidized cofactors generated in the ETC (NAD) are used to fuel glycolysis and the TCA cycle (Steuber, 2001).

Since NAD(H) cannot traverse the mitochondrial membrane in *S. cerevisiae*, the compartmentalization of glycolysis in the cytosol and the pyruvate dehydrogenase complex (PDH complex) and the TCA cycle in mitochondria requires that NADH be reoxidized in the compartment it is generated. Cytosolic NADH is oxidized by two external NADH dehydrogenases (encoded by *NDE1* and *NDE2* genes) and the glycerol-3-phosphate dehydrogenase shuttle (encoded by *GUT2* gene), which transport the electrons from NADH to

the ETC (in the mitochondria). Mitochondrial NADH is oxidized by NADH dehydrogenase (encoded by *NDI1*). Although this Ndi1 is homologous to *E. coli* NADH dehydrogenase I, it does not generate PMF, which possibly could explain the low P:O ratio observed in *S. cerevisiae* (Bakker et al., 2001). Furthermore, unlike the multi-subunit complex 1-type NADH dehydrogenase commonly found in other eukaryotes, including other yeasts, Ndi1 in *S. cerevisiae* is a single subunit. *S. cerevisiae* also lacks the cyanide-resistant alternate terminal oxidase (AOX), which provides an alternate respiratory pathway by transporting electrons directly from ubiquinol to oxygen and is activated when enzyme complex II and III are poisoned in other yeasts.

Given this background, I investigated the metabolic consequences of providing additional means of NADH oxidation in *E. coli* and *S. cerevisiae* and described the observations using high throughput analyses of transcription and metabolic fluxes to reveal regulatory mechanisms that control that are sensitive to redox changes. Specifically, I cloned water-forming NADH oxidase (encoded by *nox* gene) from *Streptococcus pneumoniae* in *E. coli* and *S. cerevisiae* as a means to enhance NADH oxidation. This enzyme converts NADH to NAD using molecular oxygen as substrate as follows.

$2NADH + \frac{1}{2}O_2 \rightarrow 2NAD + H_2O$

The impact of this genetic manipulation was studied in detail. These observations and their interpretation are described in Chapter 7, which also contains the identification of regulatory mechanism that triggers these metabolic changes. Using the newly-discovered regulatory features that apparently control aerobic acetate generation, the regulatory network of *E. coli* was engineered, based on hypothesis-guided design, resulting in a strain that is devoid of acetate

formation even under conditions that are normally conducive to accumulating acetate. Chapter 8 describes the relevance of this research to industrial applications as was established by demonstrating increased yield and productivity of recombinant β -galactosidase obtained using this metabolically engineered strain. The research described in this chapter formed the basis for applying for a provisional US patent.

Overflow metabolism manifests in *S. cerevisiae* in the form of aerobic ethanol or glycerol generation. Since the conditions under which *S. cerevisiae* generates ethanol or glycerol and *E. coli* generates acetate are analogous, the mechanisms by which ethanol or glycerol are generated by *S. cerevisiae* may be similar to that by which *E. coli* generates acetate. Moreover, I also studied the impact of cloning the alternate oxidase (encoded by *AOX* gene) from *Histoplasma capsulatum* in *S. cerevisiae*. The motivation behind cloning this gene in *S. cerevisiae* and the metabolic consequences, and the regulation of metabolism in the presence of this gene as revealed by transcription profiling experiments is given in Chapter 9.

The next chapter, Chapter 10, is devoted to a comparative analysis of structure, organization of the ETC in *E. coli* and *S. cerevisiae* and the redox regulation controlling the transfer of electrons. I also discuss the impact of providing an additional NADH sink on oxidative phosphorylation in this chapter. Finally, the dissertation ends with a summary of my contribution to the field of systems biology, the understanding of redox metabolism in *E. coli* and *S. cerevisiae* and how it regulates overflow metabolism in these organisms. I also compare the practice of "White Biotechnology" in Europe and the United States as well as comment on the future direction the field of systems biology should head.

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CHAPTER 2

OVERFLOW METABOLISM IN ESCHERICHIA COLI DURING STEADY-STATE GROWTH: TRANSCRIPTIONAL REGULATION AND EFFECT OF REDOX RATIO¹

Out, damned spot! out, I say.

- Lady Macbeth in "Macbeth" by William Shakespeare

Data! data! data! I can't make bricks without clay.

Sherlock Holmes to Watson in "The adventure of the copper beeches"

¹ Vemuri, G.N., Altman, E., Sangurdekar, D.P., Khodursky, A.B., Eiteman, M.A., Appl. Env. Microbiol., 2006 72:3653-3661 Reprinted with permission of publisher

Abstract

Overflow metabolism in the form of aerobic acetate excretion by *Escherichia coli* is an important physiological characteristic of this common industrial microorganism. Although acetate formation occurs under conditions of high glucose consumption, the genetic mechanisms that trigger this phenomenon are not clearly understood. We report on the role of the NADH/NAD ratio ("redox ratio") on overflow metabolism. We modulated the redox ratio in E. coli through the expression of Streptococcus pneumoniae (water-forming) NADH oxidase. Using steadystate chemostat cultures, we demonstrated a strong correlation between acetate formation and this redox ratio. We furthermore completed genome-wide transcription analyses of a control E. coli strain and E. coli overexpressing NADH oxidase. This transcription results showed that in the control E. coli several genes in the tricarboxylic acid (TCA) cycle and respiration were repressed as glucose consumption rate increased. Moreover, the relative repression on these genes was alleviated by NADH oxidase and the resulting reduced redox ratio. Analysis of a promoter binding site upstream of the genes which correlated with redox ratio revealed a degenerate sequence with strong homology with the binding site for ArcA. Deletion of arcA resulted in reduced acetate and increased the biomass yield due to the increased capacities of the TCA cycle and respiration. Acetate formation was completely eliminated by reducing the redox ratio through expression of NADH oxidase in the *arcA* mutant, even at a very high glucose consumption rate. The results provide the basis for studying new regulatory mechanisms prevalent at reduced NADH/NAD ratio, as well as in designing more efficient bioprocesses.

Introduction

Escherichia coli accumulates acetic acid when growing at a high rate of glucose consumption even in the presence of ample oxygen (2,11,30). This phenomenon is known as "overflow metabolism". Acetate is generated when carbon flux from acetyl CoA is directed to acetate instead of entering the tricarboxylic acid (TCA) cycle (11). This by-product induces a stress response even at extremely low concentrations (21), hinders growth (26) and reduces the production of recombinant proteins (37). Overflow metabolism has been attributed to an enzymatic limitation in the TCA cycle (27). In *E. coli* the complete oxidation of one mole of glucose in glycolysis and the TCA cycle generates 10 moles of NAD(P)H and 2 moles of FADH₂ (31):

Glucose +
$$8NAD^+$$
 + $2NADP^+$ + $2FAD$ + $4ADP$ + $4P_i \rightarrow 6CO_2$ + $8NADH$ + $2NADPH$ + $2FADH_2$ + $4ATP$ + $10H^+$

If the rate of oxygen utilization is sufficiently high, the reduced cofactors generated by glucose consumption are reoxidized in the electron transport chain (ETC), which serves the dual purpose of maintaining an optimal redox environment and generating energy by oxidative phosphorylation. In the absence of oxygen glucose cannot be completely oxidized, and metabolic intermediates accumulate to maintain the redox balance. Even in the presence of oxygen, if the rate of glucose consumption is greater than the capacity to reoxidize the reduced equivalents generated, the response is similar to what is observed under anaerobic conditions (1,2,13). Since the flux from acetyl CoA to acetate does not generate any NADH, while the flux from acetyl CoA through the TCA cycle generates 8 NAD(P)H and 2 FADH₂, carbon flow diversion to acetate could be viewed as a means to reduce or prevent further NAD(P)H accumulation (7,13). These inferences regarding acetate overflow have been based on

physiological observations and *in vitro* enzyme assays, and the genetic trigger has not been identified. Details of pathways involved in acetate generation and consumption, including specific enzymes and their regulation, have been recently reviewed (39).

Aided by genomic technology, we further investigated the relationship between redox and acetate overflow by transforming wild-type *E. coli* with water-forming NADH oxidase (encoded by *nox* gene from *Streptococcus pneumoniae*) in order to oxidize residual NADH (3). This enzyme decouples NADH oxidation (oxygen reduction) from respiratory energy generation (10). We report the impact of this perturbation in redox on physiological and transcriptional activity of cells under steady-state conditions and correlate the results with acetate formation to begin to identify regulatory processes involved in overflow metabolism. Steady-state chemostat cultures (e.g., as opposed to batch culture) are an important tool to study overflow metabolism and transcriptional changes because: 1) a chemostat permits the system to be tuned to achieve a desired glucose consumption rate through the selection of dilution rate—in particular glucose consumption rates both above and below the threshold rate which initiates acetate formation, and 2) steady-state conditions permit collection of transcriptional information which is itself at a steady-state. These results provide insights into gene regulation in *E. coli* as a model facultative anaerobe during the transition from respiratory to respiro-fermentative metabolism.

Materials and Methods

Microorganisms and Media—The *E. coli* K-12 strains MG1655 and QC2575 (MG1655 $\Delta arcA$::Tet) were used in this study. QC2575 was obtained from D. Touati (l'Institut Jacques Monod, Paris, France). Growth and physiological characteristics used defined media (8) composed of (per liter): 5 g glucose, 1.5 g NH₄Cl, 0.5 g NaCl, 7.8 g Na₂HPO₄·7H₂O, 3.5 g

KH₂PO₄, 0.014 g CaCl₂·2H₂O, 0.246 g MgSO₄·7H₂O, 0.1 mL Antifoam C, 1 mg biotin, 1 mg thiamine, 100 mg ampicillin and 10 mL trace metal solution. The trace metal solution contained (per liter): 16.68 g FeCl₃·6H₂O, 0.36 g ZnSO₄·7H₂O, 0.32 g CuSO₄·5H₂O, 0.2 g MnSO₄·H₂O, 0.18 g CoCl₂·6H₂O, 22.4 g EDTA, 0.1 g NaMoO₄·2H₂O.

Construction of pTrc99A-nox—The *Streptococcus pneumoniae nox* gene was amplified using the polymerase chain reaction using pPANOX7 as template (M.-C. Trombe, U. Paul Sabatier, Toulouse, France) with *Pfu* DNA polymerase. Primers were designed based on the published *S. pneumoniae nox* gene sequence (3) and contained a *Bam*HI restriction site and Shine-Dalgarno sequence at the beginning of the amplified fragment and a *Pst*I restriction site at the end of the amplified fragment; forward primer 5'-TAC TAT <u>GGA TCC AGG AGG T</u>AA CAG CT<u>A TG</u>A GTA AAA TCG TTG TAG TCG GTG C-3'; reverse primer 5'-ATA TAG TGA TCG ATA GCA GT<u>C TGC AG</u>T TAT TTT TCA GCC GTA AGG GCA GC-3' (the *Bam*HI, Shine-Dalgarno, ATG start, and *Pst*I sites are respectively underlined). The resulting 1.4 kb PCR product was gel isolated, digested with *Bam*HI and *Pst*I and ligated into the pTrc99A expression vector which had been digested with the same two restriction enzymes.

Chemostat cultivation—Carbon-limited chemostats of 1.5 L working volume were performed in 2.5 L vessels (Bioflo II, New Brunswick Scientific, NJ) at 37°C, pH 7.0 and an agitation of 500 rpm. Air flowrate was maintained at 1.5 L/min using mass flow controllers (Unit Instruments, Orange, CA) to ensure that the dissolved oxygen concentration remained above 40% of saturation at all growth rates studied. Measurements were made after the cells attained a steady-state, which required at least 7 volume changes without any perturbation. Biomass formed was quantified by washing the cells with phosphate buffer saline (pH 7.0) and drying for 12 h at 60°C. Glucose and organic acids in the feed and effluent were measured by HPLC with about

0.05 g/L as the detection limit (6). Oxygen uptake rate and CO₂ evolution rate were calculated by measuring the effluent concentrations of oxygen and CO₂ (Ultramat 23 gas analyzer, Siemens, Germany). Each steady-state growth condition was freshly started from a single colony.

Quantification of NADH/NAD and glycolytic metabolites—Metabolism was rapidly quenched by extracting two 10 mL aliquots from a chemostat into 40 mL methanol pre-chilled for 4h in a dry ice-ethanol bath. The cell pellets were resuspended in 0.2 M HCl (for extracting NAD) or 0.2 M NaOH (for extracting NADH), and the nucleotides were extracted by boiling the cell suspension. The cycling assay (4) which involves the transfer of reducing equivalents from NADH ultimately to 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was used to measure the specific nucleotides (23). The rate of reduction of MTT as measured at 570 nm was proportional to the concentration of NADH or NAD.

The intracellular concentrations of the key metabolites glucose-6-phosphate, fructose-6-phosphate, phosphoenol pyruvate (PEP), pyruvate and acetyl CoA were measured enzymatically using cell-free extracts prepared by the perchloric acid method (36).

Global transcription profiling—Changes in the expression of genes at various growth rates were identified using parallel two-color hybridization to whole-genome E. coli MG1655 spotted DNA arrays corresponding to 98.8% of the annotated ORFs. Detailed description of the design, printing and probing are described previously (19,20). After attaining a steady-state at a predetermined dilution rate, samples from the chemostat were extracted into RNAprotect buffer (Qiagen, Valencia, CA), and the cell pellets were frozen at -80° C. Total RNA was extracted by hot phenol-chloroform method and treated with DNase I in presence of RNase inhibitor for subsequent labeling with Cy3-dUTP and Cy5-dUTP fluorescent dyes by reverse transcription. Total RNA from the strain containing pTrc99A plasmid was cultured at a dilution rate of 0.1 h⁻¹

was used as the common reference condition (always labeled with Cy3-dUTP) against which total RNA extracted from cells cultured at five higher equally-spaced dilution rates (always labeled with Cy5-dUTP) was hybridized. Similarly, for the strain containing the pTrc99A-nox plasmid, total RNA extracted from cells cultured at a dilution rate of 0.06 h⁻¹ was the reference against which total RNA from cells cultured at five higher equally-spaced dilution rates was hybridized. Differential gene expression between the two reference conditions (NOX⁻ reference at a dilution rate of 0.1 h^{-1} , labeled with Cy3-dUTP and NOX⁺ reference at a dilution rate of 0.06 h^{-1} , labeled with Cy5-dUTP) was also measured to identify transcriptional changes due to the presence of the S. pneumoniae NADH oxidase. All the hybridizations were performed at least in triplicate using biologically independent samples as described (20) by incubating the labeled mixture on the arrays at 65°C overnight. The slide was subsequently washed and scanned using GenePix 4000B microarray scanner (Axon Instruments, Union City, CA). The degree of labeling of the two dyes was quantified by measuring the intensity at a wavelength of 532 nm (for Cy3) and 635 nm (for Cy5). The relative expression of a gene was calculated as the base-2 logarithmic ratio of the background subtracted intensity from the Cy5-channel to the background subtracted intensity from the Cy3-channel, and the resulting value was referred to as the "expression ratio".

Analysis of gene expression data—Our goal for the analysis of transcription data was to identify genetic changes that corresponded to physiological observations. Specifically, we were interested in identifying those genes whose expression was sensitive to a perturbation in the redox ratio (i.e., NADH/NAD ratio). We calculated the Pearson correlation coefficient for the expression ratio of each gene with the redox ratio for NOX⁺ and NOX⁻ as a function of specific glucose consumption rate. Only those genes whose expression ratios had a high correlation

coefficient (R > 0.9 or R < -0.9) with the redox ratio were considered for further analysis. These highly correlated (or anti-correlated) genes were classified into 22 functional categories according to Riley (31). Each functional category was tested for significant overrepresentation (p < 0.05) by using a hypergeometric distribution (15). With a priori information on the distribution of the global gene set among the 22 categories, hypergeometric distribution measures the enrichment of a functional category based on the number of genes of that particular category appearing in the cluster. The p-value for each category was calculated according to the following equation:

$$p = \sum_{x}^{N} \frac{\binom{K}{\binom{M-K}{N-x}}}{\binom{M}{N}}$$

where M is the total number of genes in the genome, x is the number of common genes, N is the total number of genes in the cluster and K is the total number of genes in the functional category.

Only those genes from the significantly enriched functional categories were selected to study common regulatory mechanisms governing their expression. Any co-regulation among these co-expressed genes was identified by searching for common transcription factor binding sites upstream of their transcription start site. Sequences 300 bp upstream of the filtered genes were analyzed for common sequence motifs, using the Hidden Markov Model-based Bioprospector software (24).

Results

Physiological response due to NADH oxidase overexpression—We used two isogenic strains MG1655/pTrc99A (NOX⁻) and MG1655/pTrc99A-*nox* (NOX⁺) that differ only in the presence of the *nox* gene. In batch cultures, NOX⁻ grew at a μ_{max} of 0.70 h⁻¹ while NOX⁺ grew more slowly at a μ_{max} of 0.51 h⁻¹. Based on these results, seven equally spaced dilution rates were selected for chemostat experiments to assess steady-state physiological and transcriptional responses to the overexpression of the *nox* gene. Both NOX⁻ and NOX⁺ exhibited fully respiratory metabolism until a critical dilution rate (or growth rate) was reached, above which respiro-fermentative metabolism was observed. The value of this critical dilution rate was about 0.4 h⁻¹ for the control NOX⁻ and about 0.3 h⁻¹ for NOX⁺. No glucose was observed in the effluent for a dilution rate of less than 0.4 h⁻¹.

As shown in Figure 2.1, acetate overflow is directly related to the rate at which the sole carbon source (glucose) is consumed, with acetate formation occurring only after glucose consumption surpasses some threshold rate. The presence of heterologous NADH oxidase had the effect of increasing the critical glucose consumption rate (q_s^{crit}) at which acetate first appeared and thereby delaying the entry of E. coli into respiro-fermentative "overflow" metabolism (Figure 2.1). This transition between respiratory and respiro-fermentative metabolism occurred at a q_s^{crit} of 0.8 g/g DCW h for NOX⁻ and 1.2 g/g DCW h for NOX⁺. The expression of NADH oxidase therefore increased by 50% the value of q_s^{crit} . During respiro-fermentative metabolism, NOX⁺ exhibited a lower effluent acetate concentration and a lower specific acetate formation rate (q_A) than NOX⁻ at any given q_s . Biomass yield ($Y_{X/S}$) from glucose (g dry cell weight/ g glucose consumed) was 0.42 – 0.48 g/g for NOX⁻ during respiratory metabolism but decreased during respiro-fermentative metabolism, consistent with a

portion of the glucose carbon being diverted from biomass synthesis to acetate formation. For NOX⁺, $Y_{X/S}$ maintained 0.28 g/g at glucose consumption rates above 0.5 g/g DCW h (Figure 2.1) Figure 0-1

Steady-state physiological profiles of *E. coli* in the presence of heterologous NADH oxidase. The biomass yield ($Y_{X/S}$; \diamond , \blacklozenge) and specific acetate formation rate (q_A ; \bigcirc , \blacklozenge) are compared for NOX⁻ (open symbols and dashed lines) and NOX⁺ (solid symbols and lines) as functions of specific glucose consumption rate. The highest dilution rate studied was about 80% of μ_{max} for both strains. The arrows indicate for each strain the critical specific glucose consumption rates at which acetate formation commenced.



The specific oxygen consumption rate (q_{02}) was twice as great for NOX⁺ compared to q_{02} for NOX⁻ at any given value of q_s (Figure 2.2), consistent with additional oxygen required for increased oxidation of NADH to NAD. NOX⁺ also yielded about a 50% greater specific CO₂ evolution rate (q_{CO2}) than NOX⁻ for any q_s (Figure 2.2), suggesting greater flux through CO₂-forming pathways (e.g., TCA cycle) for NOX⁺. The results show that in the presence of NADH oxidase, cells diverted less carbon to biomass and acetate and more carbon to CO₂ at any given rate of glucose consumption. A carbon balance for NOX⁻ was within ±8% under all conditions, while for NOX⁺ the carbon balance was within ±15% (data not shown), assuming identical biomass composition (and thus identical expression of biosynthetic genes). The redox balance closed for the NOX⁻ within ±9%, while for NOX⁺ this balance was only within ±30% (data not shown).

Figure 0-2

Steady-state respiration for NOX⁻ (open symbols and dashed lines) and NOX⁺ (solid symbols and lines). The steady-state specific oxygen uptake rate $(q_{02}; \Delta, \blacktriangle)$ and carbon dioxide evolution rate $(q_{CO2}; \nabla, \nabla)$ are shown as a function of specific glucose consumption rate (q_s) .


Intracellular response due to NADH oxidase overexpression—Since the expression of heterologous NADH oxidase in *E. coli* would be expected to influence the steady-state intracellular NADH and NAD concentrations, the concentrations of each cofactor were determined at each steady-state for both strains. For both NOX⁻ and NOX⁺, the intracellular concentration of NAD changed less than 30%, while the NADH concentration changed over tenfold between the lowest and highest glucose consumption rates. Moreover, the NADH concentration increased more quickly for NOX⁻ at lower values of q_s than for NOX⁺. For example, at a q_s of about 0.10 g/g DCW h, the NADH concentration was 0.03 µmol/g DCW for both strains, while at a q_s of about 1.0 g/g DCW h, the NADH concentration was 0.53 µmol/g

DCW for NOX⁻ but only 0.11 μ mol/g DCW for NOX⁺. These changes are reflected in the NADH/NAD ratios ("redox ratios", Figure 2.3). At any given value of qs, the redox ratio was always greater for NOX⁻ than for NOX⁺. The redox ratio remained at 0.01-0.02 for both strains during respiratory metabolism, but increased just prior to the onset of acetate overflow. Acetate formation for both strains occurred at an identical redox ratio of about 0.06 (Figure 2.3). Clearly, this critical redox ratio marked a boundary between respiratory metabolism and respiro-fermentative metabolism. These results indicate a correlation between the redox ratio and acetate formation. What remains unclear is whether acetate formation is a consequence of the cells achieving the critical redox ratio, whether the increased redox ratio is caused by acetate formation, or whether these two phenomena are independent consequences of some underlying change in metabolism when the glucose consumption rate surpasses qs^{crit}.

Figure 0-3

In vivo molar concentration ratio of NADH/NAD for the NOX⁻ (\Box) and NOX⁺ (\blacksquare) as functions of q_s. The critical value of NADH/NAD ratio at which acetate formation commences is about 0.06 for both NOX⁻ and NOX⁺ (indicated by vertical lines). Specific acetate formation rates (q_A) are also shown for NOX⁻ (\bigcirc) and NOX⁺ (\blacklozenge) as functions of q_s



We measured the steady-state intracellular concentration of key glycolytic intermediates in order to identify imbalances that might occur between glucose consumption and its subsequent metabolism. Steady-state pools of early glycolytic intermediates (glucose-6-phosphate and fructose-6-phosphate) increased with increasing q_s for NOX⁻ (Figure 2.4a). Although pyruvate concentration increased, PEP concentration decreased markedly just at the onset of acetate formation, so that the pyruvate:PEP ratio increased from about 0.95 to 25. For NOX⁺, steadystate concentrations for each metabolite were essentially identical to those for NOX⁻ at the lowest q_s . However, the balance between PEP and pyruvate did not vary much with increasing q_s (Figure 2.4b), with the pyruvate:PEP ratio increasing from about 0.90 to only 1.3.

Pyruvate and PEP in particular participate in a large number of biochemical reactions and therefore these metabolites tightly regulate a large portion of the metabolic network. The observed increase in the steady-state level of pyruvate could indicate increased fluxes in pathways using pyruvate as a substrate or as an enzyme activator, such as those pathways leading to the formation of acetate. Any shift in the pyruvate:PEP ratio suggests a shift in the degree of utilization of pathways that involve pyruvate compared to PEP. The correlation between acetate overflow and pyruvate:PEP ratio is consistent with an elevated intracellular level of pyruvate being a presage to acetate formation.

Figure 0-4

Intracellular concentrations of key metabolites in the glycolysis: glucose-6-phosphate (red), fructose-6-phosphate (green) PEP (yellow), pyruvate (blue) and acetyl CoA (pink) were measured at steady-state conditions in NOX⁻ (A) and NOX⁺ (B). Specific acetate formation rates (q_A) are also shown for NOX⁻ (\bigcirc and dashed lines) and NOX⁺ (\bullet and solid lines) as functions of q_S .



Transcriptional response to increasing glucose consumption rate—Since most physiological events originate at the transcription level, we measured the transcriptional responses to changes in q_s for NOX⁻ and NOX⁺ strains to establish a genetic basis for the observed physiological changes. For each strain, we used a low q_s (corresponding to dilution rates of 0.1 h⁻¹ for NOX⁻ and 0.06 h⁻¹ for NOX⁺) as the reference. We first compared the transcription profile between the two reference conditions (NOX⁻ grown at 0.1 h⁻¹ and NOX⁺ grown at 0.06 h⁻¹) to identify transcriptional changes only due to the presence of NADH oxidase. There were no significant transcriptional changes between these two references conditions, suggesting limited influence of NADH oxidase at low q_s . This result is consistent with the similar physiological parameters ($Y_{X/S}$, q_s , q_{CO2} and q_{O2}) and redox ratios observed between the two strains at low q_s (Figures 2.1–2.3).

Next, we compared the transcriptional changes at higher values of q_s relative to the appropriate reference condition for each strain. In general, we did not observe drastic changes in gene expression, but many genes exhibited a reproducible monotonically increasing or decreasing behavior relative to the reference as q_s or growth rate increased. For NOX⁻, the expression of 427 genes varied significantly with q_s (p<0.01), while only 47 achieved this level of significance for NOX⁺, and only 21 genes were common to both subsets. Among the genes whose expression varied significantly for both NOX⁻ and NOX⁺ were key genes in the biosynthesis of threonine, serine and nucleotides along with q_s for both strains, their expression is presumably largely glucose consumption rate dependent and relatively insensitive to the redox state of the cell. The average expression ratios of all genes involved in the central metabolic pathways for NOX⁻ and NOX⁺ relative to their respective reference condition are shown in

Figure 2.5 as a function of q_s . Transcription profiles of individual genes in these central metabolic pathways for the two strains are shown in Supplement 1.**Figure 0-5**

Transcriptional profile of central metabolic pathways for NOX⁻ (dashed lines) and NOX⁺ (solid lines). The mean values of the expression ratios are shown for all genes involved in glycolysis (green), TCA cycle (red), pentose phosphate pathway (PPP, blue) and respiratory genes (black) as a function of q_s . Vertical lines show the demarcation between respiratory and respiro-fermentative metabolism for NOX⁻ (dashed) and for NOX⁺ (solid). See Supplement 1 for detailed expression profiles of individual genes.



The expression of most of the central metabolic genes (genes of glycolysis TCA cycle, pentose phosphate pathway and respiration) increased during the respiratory phase of metabolism, but began to decrease just prior to respiro-fermentative metabolism for both NOX⁻ and NOX⁺, despite q_s^{crit} being 50% higher for NOX⁺. Regarding some of the key genes of interest to acetate formation: Isocitrate dehydrogenase (*icd*) and citrate synthase (*gltA*) are inhibited by NADH and have been implicated as controlling flux in the TCA cycle (12,38), and we also observed repression of these genes for NOX⁻ but induction for NOX⁺ as glucose consumption rate increased. Thus, these genes appear to control TCA cycle flux at two levels: through enzyme activity and transcription. Interestingly, some TCA cycle genes appear similarly to be controlled at both levels (e.g., sucC, sucD) while other TCA cycle genes (e.g., sucB, sdhC) encode for enzymes not known to be controlled by redox ratio but which show similar repression with increasing q_s . Induction of the acetate kinase gene (*ackA*) correlated with the formation of acetate for NOX⁻ while phosphotransacetylase (*pta*) expression was slightly repressed. For NOX^+ the expression of *ackA* and *pta* increased with q_S during respiratory metabolism and remained constant during respiro-fermentative metabolism (Supplement 1). The key acetate consumption gene, acs, was severely repressed in both strains (more than 5 fold) with increasing q_s . The pyruvate oxidase gene (*poxB*) was induced for NOX⁻ at low q_s while repressed at high qs. Genes involved in aerobic respiration such as the nuo operon (NADH dehydrogenase I chains) were generally repressed for NOX⁻ (Figure 2.5, Supplement 1), and this repression was relieved for NOX⁺. The relative expression of the *ndh* gene encoding for NADH dehydrogenase II, a primary source of NAD turnover under aerobic conditions, increased steadily with q_s for NOX⁻ and NOX⁺ during respiratory growth before saturating under respiro-fermentative conditions.

The relative expression of intermediate metabolic genes involved in the biosynthesis of amino acids and nucleotides increased with q_s, before either stabilizing or slightly decreasing at high q_s for both NOX⁻ and NOX⁺ (Supplement 1). Moreover, we did not observe significant differences in the expression profiles of these genes between the strains at any given value of q_s , except for those involved in methionine and glycine biosynthesis. The relative expression of metABCEHJL genes either remained steady or decreased with q_s for NOX⁻, while these genes were significantly upregulated for NOX⁺. The glycine biosynthesis genes, particularly glyA, were repressed for NOX⁻, but the repression appeared to be reduced for NOX⁺. Purine and pyrimidine nucleotide biosynthesis genes monotonically increased with q_S for both strains. We also observed a repression in most of the transport genes at high q_s for both NOX⁻ and NOX⁺. Among the genes encoding for symport or antiport proteins we found a few genes of the multifacilitator family that were upregulated (such as *yhfC*, *yhaU*, *codB*, *uraA*, *proP*) while all others (including *yjcG*, *lacY*, *gltS*, *gntT*, *dctA*, *tatC*, *melB*, *nupG*) were repressed (Supplement 1). Genes belonging to the ATP binding cassette transporters and the PEP-dependent phosphotransferase systems for the uptake of several sugars (including glucose) were also strongly repressed for both NOX⁻ and NOX⁺ as q_s increased (Supplement 1). There was no particular trend observed in most unclassified genes except b4249 (a putative oxidoreductase), which was induced for NOX⁻, but was repressed for NOX⁺ as q_s increased.

Identifying co-regulation among co-expressed genes—The approach governing our data analysis methodology was first to group co-expressed genes and then to evaluate these gene groups for common regulatory mechanisms (see Materials and Methods). Genes belonging to the biosynthesis of amino acids, co-factors, macromolecules and nucleotides along with central and intermediary metabolic genes were positively correlated with q_s (R > 0.9) for both NOX⁻ and

NOX⁺. Among the genes that were negatively correlated with q_s (R < -0.9) were those responsible for the degradation of small molecules, transport proteins and unclassified genes. Interestingly, we observed that only the genes belonging to the biosynthesis of amino acids, cofactors and nucleotides along with the central and intermediary metabolic genes were correlated (R > 0.9) with the redox ratio for NOX⁻ (Figure 2.6). There were also several partially classified genes in this sub-set, suggesting that the expression of a majority of these genes depends on the rate of glucose consumption and/or redox. While we identified strongly overrepresented sequences upstream of genes correlated with q_s, we could not relate these sequences with any of the known promoter binding sites. However, a significantly overrepresented ($p < 10^{-170}$) sequence (Figure 2.6) upstream of the genes correlated with the redox ratio for NOX⁻ was identified (by Bioprospector) as the binding site for ArcA (25,28,33). The identification of an ArcA binding site upstream of genes that were correlated with the redox ratio (for NOX⁻) is consistent with a recent discovery that cellular redox state is the signal for the activation of ArcB signal transduction (9,28). Supplement 2 provides a complete list of genes in NOX⁻ that showed a reduction in expression by high NADH/NAD (negatively correlated with redox ratio) and which were determined (by Bioprospector) to have a binding site for ArcA. In light of these results, we speculated that the strong repression observed for several TCA cycle and respiratory genes in NOX⁻ at high q_s might be relieved by deleting *arcA*

Figure 0-6

Hierarchical clustering of genes (rows) that are correlated (R > 0.9 or R < -0.9) with the redox ratio (NADH/NAD) in NOX⁻ as a function of increasing q_S (columns). Significantly overrepresented functional categories are shown in the table next to the figure, along with the number of genes in each category and the p-value of its significance as calculated using a hypergeometric distribution. Several key genes of the TCA cycle, respiration and biosynthesis exhibited a strong negative correlation with the redox ratio. A large portion of the genes negatively correlated to the redox ratio were partially classified, revealing redox-dependent regulation of many of these genes.



Characterization of arcA mutant—The identification of ArcA binding sites upstream of genes correlated with the redox ratio prompted us to characterize the phenotype of QC2575/pTrc99A (ARCA⁻NOX⁻) and QC2575/pTrc99A-*nox* (ARCA⁻NOX⁺). In batch culture, the μ_{max} for ARCA⁻NOX⁻ (0.73 h⁻¹) was similar to that for NOX⁻, but μ_{max} for ARCA⁻NOX⁺ (0.63 h⁻¹) was 20% greater than the value for NOX⁺. We performed accelerostats (15) for ARCA⁻NOX⁻ and ARCA⁻NOX⁺ to provide a pseudo-steady-state representation of physiological changes over a range of dilution rates (0.20 h⁻¹ – 0.54 h⁻¹). An accelerostat approximates a chemostat, and these experiments began at steady-state (after 7 volume changes at dilution rate of 0.2 h⁻¹). Once

acceleration rate of 0.01 h⁻². For these experiments, the yield $Y_{X/S}$ was about 30% lower for ARCA⁻NOX⁺ than for ARCA⁻NOX⁻ (Figure 2.7). The most striking result of deleting *arcA* was the absence of acetate for ARCA⁻NOX⁺ even at the highest dilution rate studied. The value of qs^{crit} for ARCA⁻NOX⁻ was 0.9 g/g DCW h, while we did not observe acetate even at value of qs of 1.5 g/g DCW h for ARCA⁻NOX⁺ (Figure 2.7). The values of q₀₂ and q_{C02} were 40% greater for ARCA⁻NOX⁻ than for NOX⁻, while they remained constant at about 27 mmol/g DCW h and 22 mmol/g DCW h respectively for ARCA⁻NOX⁺ (Figure 2.8). Although parameters obtained from a steady-state chemostat may differ from those obtained in a pseudo-steady-state accelerostat, we have similarly observed no acetate formation for ARCA⁻NOX⁺ in batch fermentations (data not shown).

Figure 0-7

Physiological characterization of ARCA⁻NOX⁻ (open symbols and dashed lines) and ARCA⁻NOX⁺ (solid symbols and lines) in accelerostat cultures. The biomass yield ($Y_{X/S}$; \diamond , \blacklozenge) and specific acetate formation rate (q_A ; \bigcirc , \blacklozenge) are compared as functions of specific glucose consumption rate. The steady-state values of these parameters obtained from chemostats for the NOX⁻ (dashed lines without symbols) and NOX⁺ (solid lines without symbols) are also shown.



Figure 0-8

Respiration of ARCA⁻NOX⁻ and ARCA⁻NOX⁻ (open symbols and dashed lines) and ARCA⁻NOX⁺ (solid symbols and lines) in accelerostat cultures. The specific oxygen uptake rate (q_{02} ; \triangle , \blacktriangle) and specific carbon dioxide formation rate (q_{C02} ; ∇ , \blacktriangledown) are compared as functions of specific glucose consumption rate. The steady-state values of these parameters obtained from chemostats for NOX⁻ (dashed lines without symbols) and NOX⁺ (solid lines without symbols) are also shown.



Encouraged by these results, we measured pseudo-steady-state gene expression in ARCA⁻NOX⁻ relative to that in NOX⁻ when both strains were grown at a specific growth rate of 0.4 h^{-1} (the critical growth rate for NOX⁻). The most important transcriptional changes in response to deleting *arcA* occurred in genes of the TCA cycle and respiration. The expression of these genes increased over 7-fold for ARCA⁻NOX⁻ (Supplement 3), presumably leading to the greater values of q_{CO2} and q_{O2} observed compared to NOX⁻. The 5-fold increase in the expression of the *ptsG* gene did not translate into increased q_s for ARCA⁻NOX⁻ relative to NOX⁻, providing further evidence that glycolysis is not transcription-limited. Interestingly, the ptsG has also been demonstrated to be under ArcA control (17). Among the 110 genes showing significant difference between the strains (p < 0.01), 30 of them are not classified while 21 are partly classified, including some regulatory genes (such as *ispH*, p = 0.008). Furthermore, we analyzed 300bp upstream of all the genes whose expression was statistically significant (p < 0.01) in ARCA⁻NOX⁻ relative to NOX⁻ for ArcA binding box. BioProspector identified a binding site for ArcA upstream of about 60% of these genes. A comprehensive list of all genes with p<0.01 is given in Supplement 3.

Discussion

The primary physiological consequences of providing additional means to oxidize excess NADH were the reduction of acetate formation and biomass yield along with a 50% increase in q_s^{crit} . Increased q_s^{crit} at the expense of biomass formation indicates faster NADH turnover (i.e., both generation and consumption). Higher q_{02} and q_{C02} values for NOX⁺ also indicate higher glycolytic and TCA cycle flux. In the current study, an increased NADH turnover due to overproduced NADH oxidase led to a 70% increase in glucose uptake at any given dilution rate

(Supplement 1), revealing a strong link between the rate of glycolysis and NAD availability. This result is in accordance with the view that control of glycolysis resides principally outside the pathway (32). A previous study (16) with ATP synthase mutants similarly increased the rate of glycolysis. More recently, increasing ATP hydrolysis by overexpressing F_1 -ATPase in *E. coli* was shown to increase the ADP pool and q_s by 70% with a concomitant reduction in $Y_{X/S}$ (22), leading to the conclusion that demand for ATP could control glycolytic flux. Our experiments overproducing NADH oxidase similarly increased q_s and also reduced the intracellular redox ratio, and *E. coli* responded by upregulating TCA cycle and PDH complex genes, pathways that synthesize NADH and generate CO₂. These results provide experimental evidence to support the theory that glycolytic flux is controlled by the cellular demand for global cofactors such as NADH or ATP.

Although the rapid generation and subsequent oxidation of NADH in the NOX⁺ strain essentially introduces a futile NAD turnover, it reveals two very important metabolic events correlated with overflow metabolism in *E. coli*: both the redox and the pyruvate:PEP ratio are correlated with the appearance of acetate. First, the redox ratio at the onset of acetate overflow was surprisingly identical for both NOX⁻ and NOX⁺ (Figure 2.3), indicating a relationship between redox state of the cell and overflow metabolism. Since numerous reactions utilize or generate NADH, the redox ratio and overflow metabolism are likely to be the consequences of a complex network of metabolic events. The importance of NADH/NAD in by-product formation in *E. coli* has been previously demonstrated through increased reduction of NAD, which resulted not only in increased acetate, but also in the appearance under aerobic conditions of typical fermentation products (5). In our study, TCA cycle genes which were generally repressed for NOX⁻ with increasing q_s commonly showed less repression upon introduction of NADH oxidase. Considering that acetate overflow has thus far been assumed to be due to rate-limiting enzymes of the TCA cycle or the ETC attaining V_{max} (1,7,13,27), our results provide evidence that acetate overflow occurs as a consequence of transcriptional repression of the TCA cycle and respiratory genes (Supplement 1). The introduction of NADH oxidase appears to delay the attainment of the critical redox ratio and limit acetate formation.

Also, the pyruvate: PEP ratio appears to be related to acetate formation (Figure 2.4). As pyruvate is the branch point between respiration and fermentation and a precursor to several macromolecules, its level is highly regulated. In E. coli, PEP is a co-substrate for glucose uptake and for the principal anaplerotic pathway during growth on glucose. Since acetate is produced from pyruvate directly (via pyruvate oxidase) or indirectly (via the PDH complex and acetate pathway), a 25-fold increase in the pyruvate: PEP ratio would shift the thermodynamic equilibria towards pyruvate utilization by these pathways. Although an increase in the pyruvate:PEP ratio may not directly cause acetate overflow, the observed shift in the control strain NOX⁻ does signal the onset of a bottleneck at the entrance to the TCA cycle, which we have shown can be modulated by redox. The introduction of NADH oxidase served to decrease the pyruvate:PEP ratio and by mass action would make acetate formation less favorable. These results provide circumstantial evidence for considering pyruvate to be one of the candidate signaling metabolites for inducing the phosphorylation of ArcB (14). NADH was previously proposed as a possible signal (14), and more recent evidence (9) indicates the cellular redox state is the signal for the activation of Arc-regulation while pyruvate is an allosteric activator (9).

The strong correlation (R > 0.9) between the redox ratio and the expression of genes belonging to central and intermediary metabolism, the biosynthesis of amino acids, co-factors and nucleotides demonstrates the important regulatory control affected by redox state. The

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identification of binding sites for the ArcA protein upstream of many of these genes suggests redox-dependent regulation of the ArcAB system, and is consistent with recent studies which proposing redox state triggers the Arc system (9). Our analysis does not rule out the possibility of secondary regulation, and therefore the relationships between redox state, ArcA and acetate overflow could be indirect. Although ArcA-mediated repression has been reported for many genes (operons) individually, their integrated effect at inducing overflow metabolism has been largely overlooked. The reduced redox ratio for NOX⁺ may delay significant activation of the Arc system. For the ARCA⁻ strains, several of the TCA cycle and respiratory genes were induced and q_{CO2} was elevated, demonstrating greater TCA cycle flux. This view is in accordance with recent C-13 studies using Arc-mutants of E. coli showing increased TCA flux under both aerobic and anaerobic conditions (34). The resulting higher rate of NADH formation appears to be accommodated at least partly by the elevated q_{O2} which results from derepression of the respiratory chain in these strains. Importantly, although the qs^{crit} was about 10% greater for ARCA⁻NOX⁻ compared to NOX⁻, acetate formation was even more pronounced for ARCA⁻ NOX^{-} at higher levels of q_{S} . One possible explanation for this observation is that the heightened TCA cycle flux resulting from the absence of ArcA-mediated repression elevated NADH accumulation to a level beyond the capacity of the (derepressed) respiratory chain. Without a transcriptional mechanism to prevent further NADH formation in the TCA cycle, acetate formation may have occurred through some other mechanism (such as inhibition of citrate synthase). The overexpression of NADH oxidase in the *arcA* strain seems sufficient to provide another outlet for NADH oxidation and prevent acetate formation even at high glucose consumption rates.

In summary, our results using steady-state chemostats support a model in which an increase in the redox ratio contributes to a repression of the TCA cycle and to acetate formation, and that this overflow is due to transcriptional limitation. Providing another outlet for NADH turnover relieves TCA cycle gene repression and delays acetate formation. An *arcA* mutation delays the onset of acetate formation (only so far) through the maintenance of both TCA cycle flux and respiration. Moreover, a strain with an *arcA* mutation *and* NADH oxidase activity appears able both to maintain an elevated TCA cycle flux and to alleviate NADH buildup, thereby preventing acetate formation altogether. Considering the deleterious impact of acetate on growth (26) and recombinant protein production (37) and the wide variety of genetic and process approaches proposed to reduce acetate formation, our findings provide evidence at the level of transcription for the cause of acetate overflow as well as offer a means to overcome it.

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CHAPTER 3

INCREASED RECOMBINANT PROTEIN PRODUCTION IN ESCHERICHIA COLI STRAINS WITH OVEREXPRESSED WATER-FORMING NADH OXIDASE AND A DELETED ARCA REGULATORY PROTEIN²

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Abstract

Glycolytic flux is increased and acetate production is reduced in *Escherichia coli* by the expression of heterologous NADH-oxidase (NOX) from *Streptococcus pneumoniae* coupled with the deletion of the *arcA* gene which encodes the ArcA regulatory protein. In this study, we examined the overproduction of a model recombinant protein in strains of *E. coli* expressing NOX with or without an *arcA* mutation. The presence of NOX or the absence of ArcA reduced acetate by about 50% and increased β -galactosidase production by 10-20%. The presence of NOX in the *arcA* strain eliminated acetate production entirely in batch fermentations and resulted in a 120% increase in β -galactosidase production.

Introduction

Acetate formation in aerobically grown cultures of *Escherichia coli* continues to be a major problem in the industrial application of this microorganism. The presence of acetate inhibits growth (Luli and Strohl, 1990; El Mansi, 2004) and the production of recombinant proteins (Jensen and Carlsen, 1990), and induces stress response even at low concentrations (Gschaedler et al., 1999). When grown on glucose, E. coli predominantly generates acetate as a result of overflow metabolism (Doelle et al., 1982; El Mansi and Holms, 1989). Acetate overflow at high glucose consumption rates is believed to result from an enzymatic limitation in the TCA cycle causing excess carbon from glycolysis to be shunted to acetate (El Mansi and Holms, 1989; Majewski and Domach, 1990) or from a saturation of the respiratory pathways used to reoxidize NADH (Andersen and von Meyenburg, 1980; Reiling et al., 1985). Since the TCA cycle generates NADH while acetate formation does not, saturation of NADH oxidation at high glucose consumption rates could cause the cell to form acetate in order to modulate the redox balance (Vemuri et al., 2005b). The saturation of specific oxygen consumption at the onset of acetate formation (Varma et al., 1993; Paalme et al., 1997) provides evidence for respiration being limiting during acetate overflow.

A large portion of the literature on *E. coli* physiology focuses on eliminating acetate formation by genetic manipulation (San et al., 1994; Chou et al., 1994; Aristidou et al., 1995) or process control (Konstantinov, 1990; Kleman et al., 1991; Riesenberg and Guthke, 1999; Åkesson et al., 2001; Johnston et al., 2003). Although these strategies reduce acetate formation, they often sacrifice cell growth rate and/or cell performance. Overexpression of anaplerotic enzymes which affect pathways that replenish the TCA cycle, also reduce acetate formation (Farmer and Liao,

1997; Gokarn et al., 2001; Vemuri et al. 2005a) and increase recombinant protein production (March et al., 2002).

Recently a strong link was demonstrated between redox ratio (in vivo molar concentration ratio of NADH/NAD) and acetate overflow in E. coli (Vemuri et al., 2005b). Specifically, the commencement of acetate overflow occurred above a critical NADH/NAD ratio of 0.06. Moreover, acetate overflow was delayed by the expression of heterologous NADH oxidase (NOX), an enzyme which served to reduce the NADH/NAD ratio at any given glucose consumption rate. The redox state has recently been reported to trigger the Arc regulon (Georgellis, 2002; Malpica et al., 2004), a two-component signaling system which represses genes of the TCA cycle and respiration. A possible model therefore is that an increased NADH/NAD ratio signals the Arc system to divert carbon to the acetate pathway as a means to reduce the further accumulation of NADH. One observation consistent with this model has been that an *arcA* deletion relieves the repression of key TCA cycle and respiration genes and reduces acetate formation (Vemuri et al., 2005b). Furthermore, increasing NADH oxidation by the expression of NOX in an arcA background completely eliminated acetate formation without reducing growth rate. Since acetate formation has been demonstrated to be inhibitory to recombinant protein production, our goal in this study was to evaluate the production of β galactosidase under various levels of acetate formation brought about by the expression of NOX and/or the deletion of *arcA*.

Materials and Methods

E. coli MG1655 was the host strain used in this study. QC2575 (MG1655 *arcA*::tet) was kindly provided by D. Touati (l'Institut Jacques Monod, Paris, France). β-galactosidase encoded by

the *lacZ* gene (Fowler and Zabin, 1978) was expressed via the plasmid pACYC184-*lacZ* (March et al., 2002), while water-forming NADH oxidase encoded by the *nox* gene from *Streptococcus pneumoniae* (Auzat et al., 1999) was expressed via the plasmid pTrc99A-*nox* (Vemuri et al., 2005b). The pTrc99A plasmid served as a control (Amann et al., 1988). Each of the four strains, NOX⁻ (MG1655/pTrc99A), NOX⁺ (MG1655/pTrc99A-*nox*), ArcA⁻NOX⁻ (QC2575/pTrc99A) and ArcA⁻NOX⁺ (QC2575/pTrc99A-*nox*) were transformed with pACYC184-*lacZ* and evaluated for β-galactosidase production.

The seed culture was started from a single colony and grown overnight at 37°C in 10 mL of Luria-Bertani broth, 1 mL of which was transferred to 500 mL shake flasks containing 100 mL of the growth medium. The growth medium contained (per liter): 10 g glucose, 5 g NH₄Cl, 0.5 g NaCl, 10 g Na₂HPO₄·7H₂O, 5 g KH₂PO₄, 0.12 g MgSO₄·7H₂O, 0.15 g CaCl₂·2H₂O, 2.5 g LB, 1 mg biotin, 1 mg thiamine, and 10 mL of a trace metal solution which contained (per L): 16.67 g FeCl₃·6H₂O, 0.18 g ZnSO₄·7H₂O, 0.16 g CuSO₄·5H₂O, 0.21 g MnSO₄·H₂O, 0.18 g CoCl₂·6H₂O, 0.10 g Na₂MoO₄·2H₂O, 0.15 g Na₂B₄O₇·10H₂O, and 22.25 g Na₂EDTA·2H₂O. All cultures contained 100 mg/L ampicillin and 20 mg/L chloramphenicol to keep selective pressure on the pTrc99A and pACYC184 plasmids, respectively. The four strains were grown in batch cultures of 2.0 L working volume in 2.5 L benchtop fermenters (Bioflow III, New Brunswick Scientific, Co., Edison, NJ) at 37°C and with an air flowrate of 2 L/min. The pH was controlled at 7.0 with NH_4OH . The impeller stirring was initially 700 rpm and was automatically adjusted to ensure that the dissolved oxygen (DO) concentration always remained above 40% saturation. Protein production was induced after 1.5 h of growth by adding IPTG to a final concentration of 1 mM. Culture samples were withdrawn from the fermenter and were stored at -20° C until subsequent analyses.

Dry cell weight (DCW) of the culture was calculated from optical density measurements at 600 nm using the correlation: $DCW = 0.4788 \times OD_{600}$, based on data from previous experiments. Residual glucose and acetate were analyzed by HPLC (Eiteman and Chastain, 1997). CO₂ and O₂ in the off-gas were measured using a gas analyzer (Ultramat 23, Siemens, Munich, Germany). Growth rate was determined by linear regression from a log plot of DCW versus time during the exponential growth phase.

Cell pellets from the samples extracted during the course of growth were resuspended in 50 mM phosphate buffer (pH 7.0) and were ruptured with a SLM-Aminco French® Pressure Cell (Spectronic Instruments, Rochester, N.Y.). The cell extract was separated from the debris by centrifugation (4°C, 8000 rpm, 10 min). The activity of NADH oxidase in the cell extract was determined at 25°C, pH 7.0 and 340 nm by measuring the disappearance of 0.3 mM NADH in the presence of 0.3 mM EDTA (Lopez de Felipe et al., 1998). One unit (U) of NADH oxidase activity converts one µmole of NADH per minute to NAD. The activity of β -galactosidase was measured as described previously (Pardee et al., 1959), where one unit of activity produced 1 nmol of *o*-nitrophenol per min at 30°C and pH 7. Total protein in the cell extracts was quantified using a BCA Protein Assay Kit (Pierce, Rockville, IL).

Results and Discussion

We previously reported that overexpressing NADH oxidase (NOX) delays the formation of acetate in *E. coli* with increasing growth rate in chemostat cultures, and that an *arcA* mutant alleviates the reduction of TCA cycle flux (Vemuri et al., 2005b). Moreover, the overexpression of NOX in an *arcA* mutant eliminates acetate accumulation, even during rapid glucose consumption (Vemuri et al., 2005b). Because three levels of acetate overflow were observed in continuous

culture (high acetate in NOX⁻, moderate acetate in NOX⁺ and ArcA⁻NOX⁻, no acetate in ArcA⁻NOX⁺), we were interested in whether the expression of a model recombinant protein in these four strains correlated with acetate formation. Thus, in this current study we evaluated these four *E. coli* strains having different respiratory capabilities for their growth, acetate formation, respiratory parameters and recombinant β -galactosidase production during batch growth.

Compared with the control strain NOX⁻, the presence of heterologous NADH oxidase in NOX⁺ increased the maximum growth rate μ_{max} , but reduced the final biomass concentration and exponential phase yield, $Y_{X/S}$ (Table 3.1, Figure 3.1). The deletion of *arcA* did not affect the growth rate, and only decreased the exponential phase biomass yield slightly. The presence of NOX in the arcA strain, ArcA⁻NOX⁺, showed the highest value for μ_{max} of 0.71 h⁻¹, while the yield was indistinguishable from NOX⁺. Irrespective of the *arcA* deletion, the specific glucose consumption rate increased more than 100% in presence of *nox* for NOX^+ and $ArcA^-NOX^+$ (Table 3.1), suggesting a need to regenerate NADH from glycolysis. By supplying the cells with a means to generate more NAD at their maximum growth rate, glycolysis—a pathway requiring NAD as a cofactor-has been hastened. A conclusion consistent with this observation is that glycolysis (originally) is limited by the availability of NAD at the maximum specific growth rate. The final concentration of acetate was 1.2 g/L in NOX⁻, between 0.6 and 0.7 g/L for both NOX⁺ and ArcA⁻ NOX⁻. We did not observe acetate accumulation in ArcA⁻NOX⁺ during batch growth (Figure 3.1). Both strains with a functional ArcA (i.e., NOX⁻ and NOX⁺) showed exponential acetate accumulation as the stationary growth phase was approached. In contrast, ArcA⁻NOX⁻ showed a decrease in the rate of acetate accumulation in parallel with a decrease in cell growth rate as the stationary phase was entered. The rate of glucose uptake was faster as a result of the presence of NOX or the deletion of *arcA* while the combined effect ArcA⁻NOX⁺ resulted in the highest rate of glucose consumption (Figure 3.1).

Table 0-1

Summary of growth and product formation in four strains of E. coli. The values of specific growth rate (μ_{max}), biomass yield from glucose ($Y_{X/S}$) and specific glucose consumption rate (q_s) were calculated during the exponential phase of growth, identified by the linear region in the ln(DCW) versus time plot.

Strain	$\mu_{max} (h^{-1})$	$Y_{X/S}\left(g/g\right)$	q _s (g/g DCW h)	Final acetate (g/L)	Final β-gal (kU/L)	NOX (U/mg)
NOX ⁻	0.55	0.48	0.27	1.21	26.8	-
NOX^+	0.66	0.29	0.60	0.63	30.0	0.50
ArcA ⁻ NOX ⁻	0.59	0.40	0.26	0.68	34.8	-
$ArcA^{-}NOX^{+}$	0.71	0.30	0.61	0.02	59.5	0.31

Figure 0-1

Growth profiles of the four strains. Glucose (\bullet) , biomass (\blacksquare) and acetate (\blacktriangle) . Each fermentation was terminated when the glucose had been consumed.



The specific oxygen uptake rate (q_{02}) reached its maximum value during the mid-exponential phase of growth for all the strains and dropped rapidly as the cells progressed into early stationary phase (Figure 3.2A). The maximum value of q_{02} was 20% higher for NOX⁺ than for ArcA⁻NOX⁺ (Figure 3.2A) while it remained at about 21 mmol/g DCW for both NOX⁻ and ArcA⁻NOX⁻. Strains containing NADH oxidase (NOX⁺ and ArcA⁻NOX⁺) consumed significantly higher oxygen compared to their isogenic control strains (NOX⁻ and ArcA⁻NOX⁻), a result consistent with greater NADH turnover in strains containing heterologous NOX. The specific CO₂ evolution rate (q_{CO2}) also achieved a maximum during the mid-exponential phase, but followed an interesting pattern in these strains. This maximum value of q_{CO2} was about 50% higher for NOX⁺ than for NOX⁻. However, an opposite trend was observed when the strain also contained the *arcA* mutation. That is, the maximum value of q_{CO2} was 50% lower in ArcA⁻NOX⁺ compared with ArcA⁻NOX⁻ (Figure 3.2B). The deletion of *arcA* increased q_{CO2} by about 50% compared to the control strain (NOX⁻). It is also interesting to note that the NOX⁺ and ArcA⁻NOX⁻ strains exhibited identical maximum values of q_{CO2} (about 25 mmol/gh) while accumulating similar final concentrations of acetate.

The reduction of fermentative/overflow behavior of *E. coli* during growth provided a very beneficial environment for the overproduction of recombinant protein (Figure 3.3). The production of the model recombinant protein, β -galactosidase, was the lowest in NOX⁻. Both NOX⁺ and ArcA⁻NOX⁻ provided a modest 10-20% increase in β -galactosidase. However, the presence of NOX in an *arcA* mutant generated well over twice the amount of β -galactosidase than in the isogenic control. Since NOX⁻ accumulated the most acetate, followed by NOX⁺, ArcA⁻NOX⁻ and ArcA⁻NOX⁺, we observed a strong (negative) correlation between overflow metabolism and the production of recombinant proteins.

Figure 0-2

Specific oxygen uptake rate (A) and specific carbon dioxide evolution rate (B) in *E. coli* strains $NOX^{-}(\bigcirc)$, $NOX^{+}(\Box)$, $ArcA^{-}NOX^{-}$, (\bullet) and $ArcA^{-}NOX^{+}(\blacksquare)$.



Figure 0-3

Production of β -galactosidase in *E. coli* strains: NOX⁻(\bigcirc), NOX⁺(\square), ArcA⁻NOX⁻, (\bullet) and ArcA⁻NOX⁺(\blacksquare).



Our results suggest that avoidance of respirofermentative metabolism is very important for protein overproduction in *E. coli*. We previously have shown that the NADH/NAD ratio and the intracellular pyruvate concentration are correlated with acetate overflow (Vemuri et al., 2005b). Pyruvate is the precursor for acetate formed either through pyruvate oxidase or through phosphotransacetylase/acetate kinase formation and has been implicated to be a potential signaling molecule in the activation of the Arc system (Georgellis et al., 1999; Rodriguez et al., 2004). Providing the cell with a means to reduce the NADH/NAD ratio while preventing the
repression of the TCA cycle by the Arc system in $ArcA^{-}NOX^{+}$ provided additional carbon to the cell for biomass and hence for protein production. The presence of NOX or the deletion of *arcA* increased the CO₂ formation, consistent with a greater flux through the TCA cycle. It is not clear, however, why the $ArcA^{-}NOX^{+}$ strain generated the least CO₂. This might be explained by a particularly high anaplerotic flux in this strain, for example, through PEP carboxylase, which by supplying TCA cycle precursors could contribute to protein production.

The experiments conducted in this present study used batch conditions in which the maximum growth rate was achieved for each given strain. Many industrial fermentations are operated under fed-batch conditions in which the growth rate is limited by the rate at which a nutrient is supplied such as the carbon source glucose. Sufficient reduction of the growth rate serves as one means to prevent acetate formation by altogether avoiding overflow metabolism. Growth of ArcA⁻ and/or NOX⁺ strains in fed-batch mode under such low growth rates would likely provide no benefit to the cell for protein production, as the cells are not displaying overflow metabolism at that growth rate. The principal affect that ArcA⁻ or NOX⁺ would have in fed-batch operation is to increase the critical growth rate or glucose consumption rate at which acetate formation commenced. Therefore, a higher nutrient feed rate and growth rate could presumably be achieved without acetate formation.

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CHAPTER 4

REDUCTION OF OVERFLOW METABOLISM IN *SACCHAROMYCES CEREVISIAE* THROUGH ENGINEERING REDOX BALANCE³

I took the one less traveled by, and that has made all the difference

> The Road not taken - Robert Frost

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Abstract

When the yeast *Saccharomyces cerevisiae* is subjected to increasing glycolytic fluxes, there is a threshold value of the glucose uptake rate at which the metabolism shifts from being purely respiratory to mixed respiratory and fermentative. This shift is characterized by ethanol production, a phenomenon known as the Crabtree effect due to its analogy with overflow of lactate in cancer cells. It is well known that at high glycolytic fluxes there is glucose repression of respiratory pathways resulting in a decrease in the respiratory capacity. However, despite many years of detailed studies on this subject, it is not known whether the onset of the Crabtree effect (or overflow metabolism) is due to a limited respiratory capacity or caused by glucosemediated repression of the respiration. In order to address this issue we increased respiration in S. cerevisiae by introducing a heterologous alternative oxidase, and observed reduced aerobic ethanol formation. In contrast, increasing non-respiratory NADH oxidation, by overexpression of a water-forming NADH oxidase, resulted only in reduced aerobic glycerol formation. The transcriptional response to elevated alternative oxidase occurred predominantly in the mitochondria, while NADH oxidase mostly affected genes that catalyze cytosolic reactions. Moreover, NADH oxidase restored the deficiency of cytosolic NADH dehydrogenases in S. These results indicate that NADH oxidase is localized in the cytosol, while cerevisiae. alternative oxidase is directed to the mitochondria, and moreover that the onset of aerobic ethanol formation is a consequence of an imbalance in the mitochondrial redox metabolism. Besides answering a fundamental scientific questions our findings have substantial interest for all biomass derived applications of S. cerevisiae, such as recombinant protein production.

Introduction

Redox homeostasis is a fundamental requirement in all biological systems for sustained metabolism and growth. The intracellular redox potential is primarily determined by the NADH/NAD ratio and to a lesser extent by the NADPH/NADP ratio. In Saccharomyces cerevisiae over 200 reactions involve these cofactors spread over a large spectrum of cellular functions (1). NADH is one of the highly connected metabolites in the metabolic network (1), and any change in the NADH/NAD ratio affects large parts of the metabolism (2). NADH is primarily generated in the cytosol during glycolysis and in the mitochondria during the TCA cycle. Since the NADH-NAD redox couple cannot traverse the mitochondrial membrane in S. cerevisiae (3), distinct mechanisms oxidize NADH to NAD in the cytosol and mitochondria. Cytosolic NADH is oxidized by two external mitochondrial membrane-bound NADH dehydrogenases encoded by NDE1 and NDE2 genes with catalytic sites facing the cytosol (4). Additionally, glycerol-3-phosphate dehydrogenase (encoded by GUT2) oxidizes cytosolic NADH with concomittant glycerol formation when the NADH formation rate surpasses its oxidation rate (5). Mitochondrial NADH is oxidized by one internal mitochondrial membranebound NADH dehydrogenase encoded by NDI1 (6).

At low specific glucose uptake rates *S. cerevisiae* has a completely respiratory metabolism of glucose. However, when the specific glucose uptake rate (or the glycolytic flux) exceeds a threshold rate there is onset of "overflow metabolism" resulting in the aerobic formation of ethanol and glycerol (7-10). This is generally believed to be due to a limitation in the capacity of the respiratory pathways (8, 11). The generation of glycolytic NADH beyond the cellular capacity for its oxidation leads to reduced conditions and ultimately reduced co-products like

ethanol and glycerol. As the fermentative pathways leading to ethanol generate less ATP than the respiratory pathway there is a need for an increased glycolytic flux, which further represses respiratory metabolism (12), and possibly further inducing overflow metabolism. Despite many years of study of this so-called Crabtree effect it is not known whether the trigger is a limitation in the respiratory capacity, onset of glucose repression of the respiratory metabolism, or simply an overflow metabolism at the pyruvate branchpoint.

Aerobic ethanol and glycerol generation is a ramification of the different capacities of fermentative and respiratory pathways (7, 13, 14). Glycerol is generated to reoxidize surplus cytosolic NADH that is formed in glycolysis (15, 16). Rapid consumption of glucose could lead to the accumulation of NADH. Therefore, decreasing NADH accumulation by elevating either the rate of respiration or the direct oxidation of NADH are logical approaches to reduce overflow metabolism in S. cerevisiae. Previous effort to reduce overflow metabolism in S. cerevisiae by manipulating redox balance include deleting the *GLR1* gene (encoding for NADPH-dependent glutathione reductase), was not successful (17). By deleting GDH1 (encoding cytosolic NADPH-dependent glutamate dehydrogenase) it was possible to slightly reduce glycerol formation (17). In another attempt to engineer the redox metabolism a combined overexpression of malic enzyme and pyruvate carboxylase resulted in increased NADPH formation at the expense of NADH and ATP formation, but there was no effect on the overflow metabolism (18). These results suggest that NADP(H) has minor role in controlling overflow metabolism in S. cerevisiae. We therefore increased the direct oxidation of NADH by overexpressing a waterforming NADH oxidase encoded by the Streptococcus pneumoniae nox gene (19) and increased respiration by overexpressing an alternative oxidase encoded by the Histoplasma capsulatum

AOX1 gene (20). The NADH oxidase decouples NADH from respiratory energy generation by converting NADH to NAD using molecular oxygen (19). The alternative oxidase mediates the cyanide-resistant, NADH-dependent transport of electrons from the ubiquinone pool to oxygen in many yeasts (21) and is uncoupled with proton translocation (22, 23). By studying the impact of these two oxidases on overflow metabolism in *S. cerevisiae* and detail the physiological and transcriptional responses to these perturbations in the redox metabolism, we demonstrate that the Crabtree effect in *S. cerevisiae* is a consequence of a limited respiratory capacity.

Materials and Methods

Yeast strains and plasmid construction - Strains and plasmids used in this study are shown in Table 4.1. The NADH oxidase gene (nox) from Streptococcus pneumoniae was PCR-amplified with pPANOX7 as template (M.-C. Trombe, U. Paul Sabatier, Toulouse, France) using Expand High Fidelity PCR system (Roche Applied Science, Indianapolis, IN). The primers were designed based on the published S. pneumoniae gene sequence, GenBank Accession number AF014458 (19), and contained a BamHI site (underlined) in the forward primer, 5'-TACGGATCCAGGAGGAACAGCTATGAGTAAAATCGTTGTAGTCGGTGC-3' (ATG start in Sall (underlined) in the primer. 5'site bold) and а site reverse ACGGGTCGACTTATTTTCAGCCGTAAGGGCAGCCA-3'.

Table 0-1

List of S. cerevisiae plasmids and strains used in this study.

Strain/Plasmid	Genotype	Reference
Plasmid		
pYX212	2μ, TPI promoter, AMP ^R	R&D Systems
pYX212-NOX	pYX212 with nox from S. pneumoniae	This study
pYX212-AOX	pYX212 with AOX1 from H. capsulatum	This study
Strain		
CEN.PK113-7D	MATa URA3 HIS3 LEU2 TRP1 MAL2-8 SUC2	P. Kötter [*]
CEN.PK113-5D	CEN.PK113-7D ura3-52	P. Kötter [*]
CEN.PK398-12B	CEN.PK113-5D <i>nde1</i> (41–1659):: <i>loxP-kanMX4-loxP nde2</i> (51–100):: <i>loxP-kanMX4-loxP gut2</i> (41–2010):: <i>loxP-kanMX4-loxP</i>	(4)
CON	CEN.PK113-5D/pYX212	This study
NOX	CEN.PK113-5D/pYX212-NOX	This study
AOX	CEN.PK113-5D/pYX212-AOX	This study
ΔΔΔ	CEN.PK263-5D/pYX212	This study
$\Delta\Delta\Delta$ -NOX	CEN.PK263-5D/pYX212-NOX	This study

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Similarly the primers for the *H. capsulatum* alternative oxidase gene (AOXI) and its flanking regions were designed based on the published gene sequence, GenBank Accession number AF133236 (20), and contained a NcoI site (underlined) in the forward primer, 5'-ATCGCCCATGGTCAGCACTGCCATTACTAATACACCTCACTTCC-3' and a SacI site (underlined) 5'in the primer, reverse TACTCGGAGCTCGTTTTGTTTAAGCTGATGCAATTTTTTGCCG-3'. The plasmid pAOX 3 1 1 was used as the template (20). The 1.4 kb nox fragment and the 1.7 kb AOX1 fragment with its flanking regions were gel isolated, digested with the appropriate restriction enzymes and ligated into the pYX212 plasmid to construct pYX212-NOX and pYX212-AOX, respectively. These three plasmids were transformed into the host strain, CEN.PK113-5D, and the yeast strains containing these plasmids are designated CON, NOX and AOX, respectively (Table 4.1). The pYX212 and pYX212-NOX plasmids were also transformed into CEN.PK398-12B, and the resulting strains are designated $\Delta\Delta\Delta$ and $\Delta\Delta\Delta$ -NOX (Table 4.1).

Media and growth conditions - The three strains (CON, NOX and AOX) were maintained on agar plates made from Synthetic Complex medium (standard dropout media) lacking uracil (SC-URA). The mineral salts media for batch and chemostat cultivations was prepared as described (24). For glucose-limited chemostat cultivations, the concentration of glucose in the feed was 10 g/L. The media for nitrogen-limited chemostats had (NH₄)₂SO₄ concentration of 1.5 g/L and was supplemented with 5.3 g/L K₂SO₄. Glucose concentration in nitrogen-limited chemostats was adjusted so that its concentration in effluent was approximately 15 g/L. Aerobic batch cultivations of 4 L were carried out as described earlier (17, 18) with initial glucose concentration of 40 g/L. At least two independent chemostat cultivations of 1 L at the desired dilution (specific growth) rate were operated as described (17, 18) and steady state was achieved when 7 volume changes occurred since the last perturbation in conditions, and the CO₂ evolution rate, O₂ consumption rate and biomass concentration remained constant during at least two volume changes ($\pm 3\%$). The critical dilution rate (D_{crit} , the specific growth rate at which ethanol formation commences) was determined for the three strains (CON, NOX and AOX) using a Climited chemostat with an online ethanol sensor and operated as a productostat (18). This sensor (Figaro TGS 822, Hammer electronic, Elsinore, Denmark) detected ethanol in the off-gas and signaled the controller to adjust the nutrient feed rate so that the effective dilution rate maintained close to D_{crit} (25). Concentrations of biomass, residual glucose and any products were measured using HPLC (18).

Enzyme activity - Enzyme activity was measured by extracting 10 mL culture into 35 mL ice, immediately centrifuging (5000 rpm at 1°C for 1 min) and storing the pellet at -80°C. For analysis, the pellet was thawed on ice, and cell free extracts prepared by lysing cells (Fastprep FP120 Savant Instruments, New York, USA) as described (26). After disruption, samples were centrifuged (20 000×g at 0°C for 20 min), and the supernatant used to determine activity. Total NADH oxidase activity was assayed spectrophometrically at 25°C in 50 mM potassium phosphate buffer (pH 7.0), 0.29 mM NADH, and 0.3 mM EDTA (27). A unit of activity was the quantity that catalyzed the oxidation of 1 µmol of NADH per min. Activities of NADH-dependent glycerol-3-phosphate dehydrogenase (28), NADH-dependent alcohol dehydrogenase (8) and NAD-dependent isocitrate dehydrogenase (29) were determined as described. Protein was quantified by the Bradford method using bovine serum albumin as a standard.

Quantification of intracellular NADH/NAD - Metabolism was rapidly quenched by extracting two 10 mL aliquots into 35 mL of methanol, pre-chilled in dry ice-ethanol bath. Cells were centrifuged (4000 rpm at –20°C for 1 min) and immediately resuspended in 0.25 mL of 0.2 M HCl (for NAD) or 0.2 M NaOH (for NADH). These suspensions were boiled for 1 min, and cell debris was removed by centrifugation (5000 rpm for 5 min). The cycling assay (30) containing 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), phenazine sulfate (PES), yeast ADHII and ethanol was used to determine the concentrations.

Transcription analysis - Global gene expression at D_{crit} was measured by growing each strain in duplicate to a steady state slightly below its respective D_{crit} , and extracting three 10 mL aliquots into ice. After immediate centrifugation (4000 rpm at 0°C for 1 min), the pellet was frozen in

liquid nitrogen and stored at -80°C. The extraction of mRNA, cDNA synthesis, cRNA synthesis and labelling, as well as array hybridisation to Affymetrix Yeast Genome 2.0 arrays (Affymetrix, Santa Clara, CA, USA) were performed as described (31). Washing and staining of arrays were performed using the GeneChip Fluidics Station 400 and scanned with the Affymetrix GeneArray Scanner. Data acquisition and quantification of array images and preliminary data analysis were performed using Microarray Suite v4.0.1 (Affymetrix, Santa Clara, CA, USA).

Identification of redox-sensitive metabolic modules - Arrays were globally scaled to a target value of 500 using the average signal from all gene features. The microarrays contain probe sets representing 9,335 distinct transcription features. After excluding all probe sets not assigned Saccharomyces yORF abbreviations identified the as in Genome Database (http://www.yeastgenome.org) and all probe sets representing groups of genes, already represented as singletons, 5650 probe sets remained. Student's t-test was used to identify genes that changed significantly between CON, NOX and AOX. Gene expression changes were mapped on the genome-scale metabolic model of S. cerevisiae (1) in order to identify metabolic hubs, based on transcriptional regulation (32). This analysis identified metabolites ("reporter metabolites") around which significant transcriptional changes occurred. The genome-scale was represented as an enzyme interaction map by representing enzymes as nodes and corresponding metabolites connecting the nodes. Those interaction modules with the maximum average score (after correcting for background distribution and size) were then identified using a simulatedannealing algorithm (32).

Results

Batch responses to engineering redox balance - Batch culture growth was compared for the control (CON), the strain overexpressing NADH oxidase (NOX), and the strain overexpressing alternative oxidase (AOX). The value of μ_{max} for NOX was 10% lower than for CON, but AOX grew at a rate indistinguishable from CON (Table 4.2). However, by-product formation differed between the strains. The ethanol yield and specific productivity were similar for CON and NOX, but were about 70% lower for AOX. In contrast, glycerol yield and specific productivity were similar for CON and AOX, but were six-fold lower for NOX (Table 4.2). Clearly, in batch cultures the NADH oxidase influences glycerol generation while the alternative oxidase affects ethanol generation. Aerobic glycerol generation in *S. cerevisiae* has previously been demonstrated to result from excess NADH in the cytosol (15, 16).

Table 0-2

Strain	μ_{max} (h ⁻¹)	Yield from glucose (g/g)			Specific	Specific rates (g/g DCW h)		
Otrain		Biomass	Ethanol	Glycerol	Glucose	Ethanol	Glycerol	
CON	0.33	0.11	0.31	0.05	0.5	0.16	0.03	
NOX	0.29	0.10	0.26	0.01	0.48	0.12	0.01	
AOX	0.34	0.09	0.08	0.05	0.68	0.04	0.02	
ΔΔΔ	0.22	0.06	0.16	0.24	0.54	0.08	0.13	
$\Delta\Delta\Delta$ -NOX	0.29	0.08	0.27	0.02	0.48	0.13	0.01	

Physiological characterization of S. cerevisiae having perturbations in redox metabolism.

All values calculated in batch culture during exponential growth phase on glucose, identified by the linear relationship between the natural logarithm of biomass and culture time.

Normally, cytosolic NADH in *S. cerevisiae* is oxidized by two external NADH dehydrogenases, encoded by *NDE1* and *NDE2* (4), or electrons are shuttled to the mitochondria by the

glyceraldehyde-3-phosphate dehydrogenase (G3PDH) shuttle, encoded by *GUT2* (33). In order to confirm that reduced glycerol formation in NOX is due to elevated oxidation of cytosolic NADH by NADH oxidase, glycerol generation in a triple deletion mutant $\Delta\Delta\Delta$ (Δ *NDE1* Δ *NDE2* Δ *GUT2*) with and without the *nox* gene was compared. The glycerol yield and the specific glycerol production for $\Delta\Delta\Delta$ were over 4-fold greater compared to CON, while for $\Delta\Delta\Delta$ -NOX glycerol generation was close to the levels observed for NOX (Table 4.2). Interestingly, $\Delta\Delta\Delta$ also exhibited low ethanol generation but $\Delta\Delta\Delta$ -NOX generated ethanol at a level similar to CON (Table 4.2).

Steady-state responses to engineering redox balance - Steady-state cultivations permitted comparative analysis of metabolic characteristics between strains at identical specific growth rates. The metabolism of CON, NOX and AOX at a specific growth rate (dilution rate) of 0.1 h⁻¹ using glucose (carbon) or ammonium (nitrogen) as limiting nutrients was studied. Under carbon limitation, the biomass yield was 10% lower for NOX and 5% lower for AOX compared to CON (Table 4.3). The presence of NADH oxidase or alternative oxidase increased the specific uptake rate of glucose (q_s) and oxygen (q_{O2}), reflecting faster glucose oxidation and subsequent metabolism. The specific CO₂ evolution rate (q_{CO2}) also was higher for NOX and AOX compared to that in CON (Table 4.3).

Table 0-3

Steady-state comparison of S. cerevisiae grown in chemostats under carbon or nitrogen limitation.

Strain	Dilution Rate (h ⁻¹)		qs ^a	Yield ^b	q _{o2} ª	qco2 ^a	q _{eth} ^a	q _{gly} ^a	Carbon Recovery
	0.10	C-limited	1.12 (0.02)	0.49 (0.00)	2.75 (0.05)	2.70 (0.00)	ΩN	ΠN	99.2 (0.8)
CON	0.27	C-limited	3.27 (0.07)	0.46 (0.01)	7.46 (0.32)	8.32 (0.22)	QN	QN	97.5 (3.2)
	0.10	N-limited	4.83 (0.32)	0.11 (0.00)	4.42 (0.35)	12.11 (0.75)	6.16 (0.70)	0.04 (0.01)	98.4 (0.5)
	0.10	C-limited	1.25 (0.00)	0.45 (0.00)	3.01 (0.04)	3.14 (0.10)	QN	ΟN	95.5 (1.8)
NON	0.26	C-limited	4.04 (0.13)	0.39 (0.01)	10.77 (0.86)	11.70 (0.58)	ND	ΟN	94.3 (0.1)
	0.10	N-limited	5.73 (0.39)	0.09 (0.00)	5.28 (0.08)	17.70 (0.99)	5.26 (0.35)	0.00 (0.00)	93.8 (1.5)
	0.10	C-limited	1.18 (0.06)	0.47 (0.00)	2.99 (0.04)	3.00 (0.02)	ΩN	ΟN	98.1 (2.8)
AOX	0.32	C-limited	3.87 (0.14)	0.43 (0.01)	9.82 (0.36)	10.92 (0.60)	QN	QN	98.6 (0.4)
	0.10	N-limited	5.27 (0.05)	0.11 (0.01)	5.54 (0.39)	17.20 (0.93)	4.21 (0.04)	0.05 (0.00)	94.1 (1.2)
^a The ul	ptake and ger	neration rates	s are given in	mmol/g DCW	/ h				
^b The y	ield is calcula	ited as the bi	iomass formed	d (in grams) fo	or a gram of gli	ucose consume	od.		
ND No:	t Detected))				

ND Not Detected The values represent the averages from two independent chemostats and the standard deviation is given in parenthesis.

Nitrogen-limited chemostats allowed study of the strains at steady-state under glucose-repressing conditions. These typically respiro-fermentative conditions were accompanied by the production of ethanol and glycerol, both not produced in the carbon-limited cultures at this low dilution rate. In the presence of NADH oxidase or alternative oxidase, the values of q_s and q_{02} were both greater, reflecting higher rates of glucose oxidation. Similar to results for the batch conditions, the specific ethanol production rate was elevated for AOX but only slightly for NOX, while NOX produced 80% less glycerol (Table 4.3). The q_{02} was 20% greater, while the q_{C02} was 40% higher for both NOX and AOX compared to CON under nitrogen-limiting conditions.

Effect of engineering the redox balance on the critical dilution rate – Steady-state experiments at the low dilution rate of 0.1 h⁻¹ under nitrogen limitation demonstrated that NADH oxidase or alternative oxidase affects the formation of ethanol and glycerol. The significant differences prompted us to determine the critical dilution rates (D_{crit}) for each strain under carbon limitation (the specific glucose uptake rate at the critical dilution rate represents the glycolytic flux at which there is onset of overflow metabolism). There was no significant difference in D_{crit} for CON (0.29 ± 0.01 h⁻¹) and NOX (0.27 ± 0.02 h⁻¹) averaged over the final 10 h of cultivation (Table 4.3). However, the D_{crit} for AOX was measured to be 0.32 ± 0.007 h⁻¹, a 10% increase compared to CON. The increase in the D_{crit} indicates higher respiratory capacity of *S. cerevisiae* in the presence of the alternative oxidase.

The three strains were each grown at a specific growth rate slightly lower than the respective D_{crit} to ensure completely respiratory conditions (Table 4.3). At this dilution rate, physiological differences between the three strains were more evident than at the dilution rate of 0.1 h⁻¹.

Compared to the lower dilution rate, NOX exhibited a 14% lower biomass, while biomass for CON and AOX was only 7% lower. Moreover, the values of q_s , q_{O2} and q_{CO2} were 3-fold greater for CON and almost 3.5-fold greater for NOX and AOX at D_{crit} compared to a dilution rate of 0.1 h⁻¹. The presence of either of the two oxidases increased the capacity of respiratory pathways by 25%, as reflected in the increase in the value of q_s and q_{O2} (Table 4.3).

Enzymatic analysis of the response to engineering redox balance – Since physiological changes in response to the introduction of NADH oxidase or alternative oxidase occurred in glycerol and ethanol production, the activities of key redox-dependent enzymes (glycerol-3-phosphate dehydrogenase, G3PDH, alcohol dehydrogenase, ADH, and isocitrate dehydrogenase, ICDH) were measured under carbon-limitation at a dilution rate of 0.1 h⁻¹ and at the D_{crit} for CON, NOX and AOX. An important difference observed was that generally the activities of cytosolic G3PDH and ADH were greater at D_{crit}, while the activity of mitochondrial ICDH was greater at a dilution rate of 0.1 h⁻¹ (Figure 4.1). Moreover, the enzyme activities followed the product formation profile in the three strains at D_{crit}. The G3PDH activity was 60% lower in NOX and 16% lower in AOX than in CON, and correlated with the lowest glycerol generation in NOX. The ADH activity was lower for both NOX and AOX compared to the CON, while ICDH activity was 60% greater for NOX and doubled for AOX (Figure 4.1).

Figure 0-1

Specific activities (in U/mg protein) of NADH-dependent glycerol-3-phosphate dehydrogenase (G3P DH), NADH-dependent alcohol dehydrogenase (ADH) and NAD-dependent isocitrate

dehydrogenase (IDH) in CON, NOX and AOX during carbon-limited chemostats grown at a dilution rate of 0.1 h^{-1} (open bars) and at respective critical dilution rate (black bars).



The NADH oxidation capacity and the intracellular NADH and NAD concentrations at steadystate were also measured. The assay for determining the NADH oxidation is not specific either for NADH oxidase or for alternative oxidase and includes native activity (e.g., NADH dehydrogenases) that *S. cerevisiae* possesses. Under carbon-limitation, this basal level of total NADH oxidation in CON was almost twice as high at D_{crit} compared to a dilution rate of 0.1^{-1} . However, under nitrogen-limitation this basal level was 50% lower at D_{crit} (Figure 4.2A). NOX and AOX consistently exhibited greater NADH oxidation activity than CON under all conditions. Interestingly, unlike in CON, the activity was not lower at D_{crit} under nitrogenlimitation in NOX and AOX (Figure 4.2A). Generally, the intracellular concentrations of NADH

and NAD correlated with the total NADH oxidation activity. Specifically, for either carbonlimited or nitrogen-limited conditions, the NADH/NAD ratio was 20–50% lower for NOX and AOX than measured for CON (Figure 4.2B).

Transcription-based identification of metabolic modules – Analyzing the genome-wide transcription response of CON, NOX and AOX provides a genetic basis for the physiological changes observed at D_{crit} . When the three strains were grown in duplicate cultures at dilution rates just below their respective D_{crit} (Table 4.3) 229 genes (for NOX) and 389 genes (for AOX) exhibited differential expression compared with CON (p-value <0.01). The products of the genes exhibiting differential expression in NOX and AOX relative to CON had functions which included carbohydrate metabolism, amino acid biosynthesis, cellular functions and stress response. The transcriptional changes were superimposed over the metabolic network to identify metabolic units that changed in response to the overexpression of the two oxidases

Figure 0-2

Evaluation of redox metabolism during chemostat cultivations under carbon or nitrogenlimitation in CON, NOX and AOX. A. Total specific NADH oxidation activity. B. Ratio of intracellular NADH/NAD. Carbon-limited chemostats were operated at a dilution of 0.1 h^{-1} (white bars) or at the respective critical dilution rate (gray bars), and the nitrogen-limited chemostat (black bars) was operated at a dilution rate of 0.1 h^{-1} .



Using an algorithm that detects metabolic modules based on biologically significant changes in gene expression (32), "reporter metabolites" were identified around which significant coordinated gene expression changes occur. The top reporter metabolites were cytosolic NAD(H) when NADH oxidase was overexpressed and mitochondrial NAD(H) when alternative

oxidase was overexpressed (Table 4.4). Additionally, several key metabolites in glycerol synthesis, fatty acid metabolism and amino acid transport were also significantly affected by overexpression of NADH oxidase. For alternative oxidase overexpression, several metabolites belonging to mitochondrial processes such as the TCA cycle, respiration and acetaldehyde metabolism were affected (Table 4.4). Differences in the localization of the reporter metabolites clearly indicate compartment-specific functionality of the two oxidases: NADH oxidase predominantly affects the cytosol, and alternative oxidase is active in the mitochondria.

The genome-scale model of *S. cerevisiae* (1) was expressed as an enzyme interaction graph where enzymes were represented as nodes with the common metabolites connecting them. The metabolic modules which had the highest score of interaction (after correcting for background distribution) indicates the metabolic subnetwork where maximal transcriptional differences occurred. In general, the overexpression of NADH oxidase caused upregulation of genes whose products catalyze the synthesis of NADH in the cytosol. For example, the induction of genes involved in the synthesis of NADH via the formate dehydrogenase pathway was observed, but significant reduction in the glycerol and ethanol synthesis pathways (Figure 4.3). The glycerol assimilation pathway, involving *GUT1* and *GPD1* genes, was upregulated while the synthesis pathway was repressed upon overexpressing NADH oxidase.

Table 0-4

Reporter metabolites around which most significant gene expression changes occurred in response to overexpression of NADH oxidase or alternative oxidase in *S. cerevisiae*.

Reporter metabolites in response to NADH oxidase	p-value ^a	Reporter metabolites in response to alternative oxidase	p-value ^a
NADH	5.38E-09	NADH - mitochondrial	7.48E-08
NAD	2.18E-08	NAD - mitochondrial	6.20E-05
sn-Glycerol 3-phosphate	3.16E-03	Orotate	1.37E-03
3-Phosphonooxypyruvate	3.98E-03	CoA - mitochondrial	1.53E-03
NADH - mitochondrial	5.17E-03	Oxaloacetate - mitochondrial	2.23E-03
Acetaldehyde - mitochondrial	6.06E-03	Acetaldehyde - mitochondrial	2.50E-03
3-Phospho-D-glyceroyl phosphate	9.18E-03	Oxygen - mitochondrial	2.85E-03
Acetaldehyde	9.50E-03	2-Nonaprenyl-3-methyl-6-methoxy- 1,4-benzoquinone - mitochondrial	2.85E-03
Myristic acid	1.00E-02	2-Phospho-D-glycerate	4.40E-03
Palmitate	1.00E-02	Glyoxylate	4.62E-03
Stearate	1.00E-02	Ubiquinone-9 - mitochondrial	4.74E-03
CYS _{xt}	1.20E-02	H^+ - mitochondrial	6.58E-03
NAD - mitochondrial	1.48E-02	Malate	7.28E-03
(3S)-3-Hydroxyacyl-CoA	1.49E-02	Orotidine 5'-phosphate	8.08E-03
Glycerone phosphate	1.78E-02	Isocitrate	9.08E-03

^a The p-value indicates the probability that normalized transcription activity around a metabolite

is the same as that in the background.

Figure 0-3

Analysis of transcription responses of NOX compared with CON superimposed on metabolic network, as described earlier (32). The key enzymes that were identified in the central metabolism as part of the subnetwork are shown in the figure.



Interestingly, the pathway leading from acetaldehyde to ethanol was repressed while the conversion of acetaldehyde to acetate was stimulated (Figure 4.3). Genes in the native pathway for NADH oxidation were severely repressed, suggesting higher affinity of bacterial NADH oxidase towards NADH than the two native NADH dehydrogenases. In contrast to NADH oxidase, the overexpression of alternative oxidase had more pronounced effects in the mitochondria (Figure 4.4). Several genes in the TCA cycle and in mitochondrial processes were significantly induced. Furthermore, genes involved in ethanol generation from acetaldehyde

(*ADH1*, *ADH2* and *ADH3*) were repressed. Alternative oxidase overexpression repressed genes in the glyoxylate shunt and fatty acid oxidation pathway. The native NADH oxidation pathway in *S. cerevisiae*, mediated by *NDI1* gene, was repressed in the presence of alternative oxidase. Gluconeogenesis genes were generally repressed when either of the oxidases was overexpressed (Figure 4.4).

Figure 0-4

Analysis of transcription responses of AOX compared with CON superimposed on metabolic network, as described earlier (32). The key enzymes that were identified in the central metabolism as part of the subnetwork are shown in the figure.



Discussion

We studied the metabolic and transcriptional responses in *S. cerevisiae* to the overexpression of either NADH oxidase or alternative oxidase. NADH oxidase mediates the non-respiratory dissipation of NADH while alternative oxidase recruits parts of the respiratory system in the transfer of electrons from NADH to oxygen. In general, physiological differences due to overexpression of either of the two oxidases were more prominent at D_{crit} since at lower dilution rates (0.1 h⁻¹) there is no overflow and presumably no "excess" NADH. The reduction in biomass yield as a result of overexpression of either of the two oxidases is likely due to increased energy dissipation. Neither of the enzymes is coupled with proton translocation, and each therefore dissipates free energy captured in NADH oxidase nor alternative oxidase was repressed by glucose as indicated by the high activity for NOX and AOX in the presence of excess glucose (Figure 4.2). Since native *S. cerevisiae* genes involved in respiration are repressed by glucose (34), these two heterologous oxidases offer a potential solution to maintaining high respiratory capacity even in the presence of high glucose concentrations.

NADH oxidase primarily impacted glycerol production, while alternative oxidase affected ethanol production. Normally, cytosolic NADH is reoxidized by two external NADH dehydrogenases, (15, 16), and when glycolytic NADH generation surpasses the rate at which these dehydrogenases can act, *S. cerevisiae* activates the glycerol synthesis pathway as another outlet for NADH consumption (33, 35). The reduced glycerol generation in NOX in batch (Table 4.2) and continuous (Table 4.3) cultures as well as the reduced G3PDH activity (Figure 4.1) demonstrates that NADH oxidase relieves the need to activate the glycerol pathway.

Moreover, the substantially reduced glycerol generation in $\Delta\Delta\Delta$ –NOX illustrates that bacterial NADH oxidase could functionally replace the native NADH dehydrogenases and is predominantly localized in the cytosol in *S. cerevisiae*. The cytosolic localization of NADH oxidase was further confirmed by identifying cytosolic NAD(H) as the top reporter metabolite around which a majority of the transcription changes occurred (Table 4.4). Furthermore, transcription analysis revealed that *GUT1* and *GPD1* genes were induced and *GUT2* was repressed in NOX relative to CON, suggesting an induction of the glycerol consumption pathway in response to NADH oxidase overexpression (Figure 4.3).

Overexpressing alternative oxidase reduced ethanol generation. Transcription analysis revealed that the overexpression of alternative oxidase induced almost every step of the TCA cycle (Figure 4.4). A similar increase in the TCA cycle activity and amino acid biosynthesis has recently been reported from proteome data when the alternative oxidase gene (Ha*AOXI*) from *Hansenula anomala* was overexpressed in *S. cerevisiae* (36). Moreover, the heterologous alternative oxidases from plants expressed in various yeasts were directed to the mitochondria (21, 37). Mitochondrial expression of alternative oxidase in this work is illustrated by the identification of mitochondrial NAD(H) and quinones as the top reporter metabolites as well as several other metabolites of mitochondrial origin around which coordinated transcriptional changes in metabolic subnetworks in the mitochondria in AOX (Figure 4.4).

Aerobic ethanol formation in *S. cerevisiae* is due to a limitation in electron transport from NADH to oxygen (9), and is accompanied by reduced activities of glycolytic enzymes

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phosphoglycerate kinase, enolase, triosephosphate isomerase (7), ethanol assimilating enzymes such as NAD-dependent alcohol dehydrogenase, NAD-acetaldehyde dehydrogenase (8) as well as an increased activity of pyruvate decarboxylase (7). The presence of alternative oxidase increased respiratory capacity by elevating the rate of NADH oxidation and q_{CO2} , and thereby facilitating greater coupling between glucose oxidation and respiratory pathways. The 2-fold increase at D_{crit} in the activity of NADH-generating ICDH (Figure 4.1) and the upregulation of several TCA cycle genes (Figure 4.4) demonstrate greater capacity of the TCA cycle in AOX compared with CON. In the absence of alternative oxidase (CON), the activity of ICDH decreased as the growth rate approached D_{crit}, an effect likely due to inhibition caused by increasing NADH/NAD ratio, as previously observed for Yarrowia lipolitica (38). Allosteric inhibition of pyruvate dehydrogenase (39, 40) and of other key TCA cycle enzymes (ICDH, α ketoglutarate dehydrogenase and malate dehydrogenase) by NADH (or by NADH/NAD ratio) restricts the entry of glycolytic carbon from pyruvate into the mitochondria. Under these circumstances carbon is shunted to acetaldehyde and subsequently to ethanol by coordinated action of pyruvate decarboxylase and NAD-dependent aldehyde dehydrogenases, routes which avoid additional NADH accumulation. When alternative oxidase activity, and thus another NADH-ubiquinol sink, was provided, this restriction in the TCA cycle was relieved. The resulting increased capacity of the TCA cycle permitted accommodation of more glycolytic carbon to enter the TCA cycle, and reduced the diversion of carbon to ethanol. Thus, our results clearly show that the onset of overflow metabolism towards ethanol is due to a limited capacity of respiratory system involved in oxidation of mitochondrial NADH.

Previously, it was shown that fusing Hxt7 (high-affinity hexose transporter) with Hxt1 (lowaffinity hexose transporter) reduced aerobic ethanol generation by modulating q_s (41). This not only resulted in decreased q_s , but also decreased the specific growth rate. In this study, we demonstrated that by engineering the redox balance of the cytosol and the mitochondria independently through NADH oxidase and alternative oxidase, it is possible to the control byproduct formation without sacrificing the rate of glucose consumption or specific growth rate. Increased non-respiratory assimilation of NADH in the cytosol by NADH oxidase creates a futile cycle which reduces glycerol generation. Increased alternative oxidase and in the mitochondria reduces ethanol generation. The introduction of these two pathways in *S. cerevisiae* therefore offers viable metabolic engineering strategies to modulate aerobic by-product formation, and in particular this will have substantial impact on the use of *S. cerevisiae* for the production of recombinant proteins where the rate of production often is limited by the presence of overflow metabolism towards ethanol.

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CHAPTER 5

REDOX HOMEOSTASIS AND BIOENERGETICS IN FACULTATIVE ANAEROBES: REGULATION AND OVERFLOW METABOLISM IN *ESCHERICHIA COLI* AND *SACCHAROMYCES CEREVISIAE*⁴

It is necessary that the reasoner should be able to utilize all the facts which have come to his knowledge; and this in itself implies, as you will readily see, a possession of all knowledge, which, even in these days of free education and encyclopaedias, is a somewhat rare accomplishment.

Sherlock Holmes to Watson in "The Five Orange Pips"

Our similarities are different

- Dale Berra

⁴ Vemuri, G.N.^{a,b}, Eiteman, M.A.^a, Olsson, L.^b, Nielsen, J.^b, To be submitted to FEMS Microbiol. Rev.

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Abstract

Microorganisms satisfy their carbon and energy requirements by oxidizing sugars with a concomitant conversion of NAD to NADH. The reoxidation of NADH to NAD occurs by the transfer of electrons to oxygen by the electron transport chain, which culminates in the generation of ATP. The intertwined nature of redox homeostasis and energy generation is universally conserved and governs growth and product formation. The formation of anaerobic products under fully aerobic conditions during rapid glucose consumption rate by facultative anaerobes is known as overflow metabolism, and is believed to be a consequence of saturation in electron transport chain. The product of overflow metabolism is acetate in *Escherichia coli*, while it is ethanol or glycerol in *Saccharomyces cerevisiae*. We analyze the structure and organization of metabolic pathways in these two organisms, and highlight the differences in their genetic makeup, redox homeostasis and energy generation to understand the distinction in regulation that lead to different overflow metabolites. We also review recent work on engineering the redox balance in *E. coli* and *S. cerevisiae* and based on these results, present a model for overflow metabolism in these two organisms.

Introduction

Redox homeostasis is an essential requirement for sustained metabolic performance in all organisms, including carbohydrate uptake and its subsequent catabolism. The reduced cofactors (such as NADH) generated in carbohydrate uptake are reoxidized by respiration in the electron transport chain (ETC), where electrons are transported to oxygen in a cascade of steps which culminate with the generation of ATP. Therefore, the ETC serves the dual roles of maintaining redox balance as well as energy generation. In the absence of oxygen, the reduced cofactors are oxidized in the fermentation reactions and generate organic acids such as lactate, acetate, formate, etc or ethanol. ATP generation under anaerobic conditions occurs by substrate-level Facultative anaerobes can change their metabolism efficiently adapt to phosphorylation. different levels of oxygen availability and combine ETC as well as fermentation pathways to maintain optimal redox balance and generate energy. The degree of utilization of the respiratory pathways (ETC) and the fermentation pathways depends not only on the level of aerobicity, but also on their respective pathway capacities, the rate of carbohydrate oxidation and the rate of generation of reduced cofactors. Despite the superficial similarities in carbohydrate metabolism, NADH oxidation and ATP generation across all species, the regulation of these pathways greatly differs between organisms and governs the metabolism and physiology. We study the differences in the regulation of these pathways and their consequence on product formation and physiology between Escherichia coli and Saccharomyces cerevisiae. These organisms serve as a model prokaryote and a model eukaryote, respectively, in many academic enquiries as well as industrial applications. The key aspect that differentiates these organisms is the compartmental organization of metabolism.
During rapid glucose uptake, even under ample oxygen availability, *E. coli* and *S. cerevisiae* generate fermentation products [1,2]. When the generation of NADH resulting from glucose oxidation exceeds the rate at which is can be oxidized, there is an overflow of carbon in glycolysis [3,4]. This carbon is shunted to fermentation products, and the phenomenon is known as overflow metabolism [2]. The product of overflow metabolism is acetate in *E. coli* and ethanol or glycerol in *S. cerevisiae*. The differences in the regulation of the central carbon metabolism and the compartmental organization of metabolism in *S. cerevisiae* possibly account for the different product formation in overflow metabolism. We examine the differences in regulation of carbohydrate metabolism in *E. coli* and *S. cerevisiae* to understand the differences in the nature of overflow metabolism in these two organisms.

Genetic comparison of E. coli and S. cerevisiae central metabolism

In order to understand the differences and in carbohydrate metabolism and subsequent physiology, it is necessary to consider the cellular genetic and metabolic organization in these two microorganisms, particularly central metabolism wherein most reduced cofactors are generated. In this section, we will briefly compare the function of the genes involved in the uptake of glucose and its subsequent processing in *E. coli* and *S. cerevisiae*.

Glucose uptake and metabolism

Glucose uptake mechanisms are highly regulated in both *E. coli* and *S. cerevisiae*. However, the processes employed by these two organisms to transport glucose from extracellular space into the cell are quite different. *E. coli* possesses a coordinated complex of enzymes that work to sense the presence of glucose and facilitate its transport into the cell. This system, known as the

phosphoenolpyruvate phosphotransferase system (PEP:PTS), transports glucose with concomitant phosphorylation using PEP as the phosphate donor [5]. The high free energy of hydrolysis of the PEP phosphoryl group (about -14.7 kcal/mol) is harnessed for both translocating and phosphorylating glucose [6]. Enzyme I (EI), encoded by *ptsI*, and histidine protein (HPr), encoded by *ptsH* (Table 5.1), are the key components of the PTS common for all hexoses, and they play a crucial role in the transfer of the phosphate group from PEP to the sugar [7]. Enzyme II (EII) is specific for the particular hexose. For example, two membrane bound proteins, B and C, and one soluble protein, IIA^{Glc} mediate glucose transport [8]. Upon simultaneous transport and phosphorylation, the glucose-6-phosphate proceeds through glycolysis. In contrast to the active sugar transport system in E. coli, S. cerevisiae uses facilitated transport for glucose uptake, and about 18 hexose transport proteins are devoted to this purpose [9]. In order to adapt to varying glucose concentrations two distinguishable uptake systems exist which differ in their kinetic affinity towards glucose. A low affinity transport system (K_m of 10-50 mM for glucose) dominates at high glucose concentrations and a high affinity system (K_m of 1-3 mM for glucose) is used at low glucose concentration [10,11]. The high and low affinity transport systems coordinate with each other to mediate the efficient glucose transport into the cell at all concentrations. Once transported into the cell, glucose is phosphorylated by two hexokinases I and II (encoded respectively by HXK1 and HXK2) and by glucokinase (GLK1) which specifically phosphorylates glucose (Table 5.1). After the concerted action of the hexose transporters and hexokinases, glucose-6-phosphate proceeds through glycolysis.

Table 0-1

List of genes involved in the uptake and metabolism of glucose in *E. coli* and *S. cerevisiae*. The reactions include glucose uptake, glycolysis, pentose phosphate pathway and the TCA cycle. The gene list is compiled using Ecocyc (http://www.ecocyc.org) and Saccharomyces Genome Database (http://www.yeastgenome.org).

	Ge	nes	Commont
Reaction	E. coli	S. cerevisiae	Comment
Sugar Uptake			
GLUex + PEP -> G6P + PYR	ptsH, ptsI, ptsG		Transport and phosphorylation
GLUex -> GLU		HXT1-17	Facilitated transport
GLU + ATP ->G6P + ADP	glk	HXK1, HXK2, GLK1	Phosphorylation
EMP pathway G6P -> F6P F6P -> F1,6dP F1,6dP ->DHAP+GAP DHAP <-> GAP	pgi pfkA, pfkB fbaA, fbaB tpi	PGI1 PFK1, PFK2 FBA1 TPI1	
GAP +NAD -> 1,3dPG + NADH	gapA, gapB	TDH1, TDH2, TDH3	
1,3dPG+ADP ->3PG+ATP 3PG -> 2PG 2PG -> PEP PEP-ADP -> PYR + ATP	pgk pgml, gpmA, gpmB eno pykA, pykF	PGK1 GPM1 ENO1, ENO2 PYK2, CDC19	
PYR + NAD -> AcoA + NADH + CO2	aceE, aceF, lpd	PDA1, PDA2, I AT1_I PD1	
Entner-Doudoroff pathway			
G6P + NADP -> d6PGL + NADPH d6PGL -> 6PdG 6PdG -> 2k3d6PG 2k3d6PG -> GAP + PYR	zwf pgl edd eda	ZWF1 SOL3, SOL4 - -	ED pathway has not been conclusively identified in <i>S. cerevisiae</i>
Pentose Phosphate pathway			
6PdG + NADP -> RI5P + NADPH + CO2	gnd	GND1, GND2	
RI5P -> X5P + R5P X5P + R5P -> S7P + GAP S7P + GAP -> E4P + F6P	rpe tktA, tktB talA, talB	RPE1 TKL1, TKL2 TAL1	
Pyruvate transport	-	YIL006	Transport of pyruvate from cytosol into the mitochondria
	-:/ 4 A		
ACOA + OAA -> CII CIT -> ICT	gitA acnA, acnB	ACO1, YJL200	

ICT + NAD(P) -> aKG + NAD(P)H	icd	IDH1, IDH2	NADP is the cofactor in <i>E. coli</i> , while <i>S.</i> <i>cerevisiae</i> uses NAD
aKG + NAD -> SuccoA + NADH	sucA, sucB, lpd	KGD1, KGD2	
SuccoA + ADP -> SUC + ATP	sucC, sucD	LSC1, LSC2	
SUC -> FUM	sdhA, sdhB, sdhC, sdhD	SDH1, SDH2, SDH3, SDH4	Complex 2 in the respiratory electron transport
FUM -> MAL	fumA, fumB, fumC	FUM1	
MAL + NAD -> OAA + NADH	mdh	MDH2	

Catabolism of glucose-6-phosphate

The Embden-Meryerhoff-Parnas (EMP) pathway is the principal pathway by which glucose-6phosphate (G6P) is metabolized in *E. coli* and *S. cerevisiae*. This pathway generates NADH and ATP in the conversion of G6P to pyruvate. The net reaction sequence can be summarized as

 $G6P + 3ADP + 2NAD + 3P_i \rightarrow 2Pyruvate + 3ATP + 2NADH$

The genes involved in the EMP pathway share a high degree of homology between *E. coli* and *S. cerevisiae*. Glyceraldehyde-3-phosphate dehydrogenase catalyzes the first committed step in the synthesis of PEP (Table 5.1). This reaction also generates one mole of NADH for every mole of glyceraldehyde-3-phosphate oxidized. Unlike most other prokaryotes, *E. coli* has two genes that encode for this enzyme, *gapA* and *gapB*. Of these two, *gapA* participates in glycolysis and has higher homology to eukaryotic sequence, while the function of *gapB* is not clearly understood [12,13]. Three genes, *TDH1*, *TDH2* and *TDH3*, are linked to glyceraldehyde-3-phosphate dehydrogenase in *S. cerevisiae* [14,15] although only *TDH2* and *TDH3* contribute to glycolysis during exponential growth while *TDH1* is believed to be active during stationary phase, indicating alternate roles for this gene [16]. The conversion of 3-phosphoglycerate to 2-phosphoglycerate is catalyzed by phosphoglycerate mutase. This enzyme is encoded by three genes, *gpmA*, *gpmB* and *pgmI* in *E. coli* [17], while only *GPM1* encodes for this enzyme in *S. cerevisiae* [18,19]. More recently, two other genes (*GPM2* and *GPM3*) have been discovered in

S. cerevisiae that share homology with GPM1, but since neither complements a GPM1 mutation, they appear to be non-functional homologs [20]. The evolutionarily unrelated E. coli genes gpmA and gpmB are both induced by glucose and have an equal role in the catalysis, while pgmI resembles the corresponding eukaryotic enzyme [17], making the metabolic significance of these isozymes unclear. E. coli has only one gene (eno) encoding an enzyme that functions reversibly. S. cerevisiae has two isozymes of enolase encoded by ENO1 and ENO2 which respectively convert 2-phosphoglycerate to PEP and the reverse reaction (Table 5.1). Recently, the E. coli enolase gene has been found to be a part of the degradosome, a complex system involved in the degradation of mRNA [21]. Enolase degrades *ptsG* mRNA in response to high levels of glucose-6-phosphate, illustrating a feedback response of this pathway in the transcriptional control of glycolysis [22]. No such regulatory role has been shown for S. cerevisiae enolases. The final glycolytic step of converting PEP to pyruvate with the formation of ATP is mediated by pyruvate kinase. In E. coli the genes pykF and pykA encode respectively for pyruvate kinase I and pyruvate kinase II which differ in their kinetic and chemical properties with respect to their sensitivity to ATP and are not interconvertible (Table 5.1). The corresponding enzyme in S. cerevisiae is also encoded by two genes, CDC19 and PYK2. While CDC19 is involved in the cell division cycle [23] and is activated by fructose-1,6-bisphosphate (similar to the E. coli pyruvate kinase I), PYK2 is subject to glucose repression [24], indicating that CDC19 (also known as *PYK1*) is the main pyruvate kinase in *S. cerevisiae* during glucose meabolism.

There are two other routes by which G6P can be converted to pyruvate. The first route is the Entner-Doudoroff (ED) pathway where G6P is cleaved by an aldolase into two 3-carbon intermediates, pyruvate and glyceraldyhyde-3-phosphate (Table 5.1). The ED pathway is less

efficient in glucose oxidation compared to the EMP pathway since only two moles of ATP are produced per mole of G6P oxidized [25]. A recently concluded evolutionary assay showed that this pathway precedes EMP pathway [26]. Curiously, S. cerevisiae is not reported to have a functional ED pathway, although genome sequence has revealed two unclassified genes with unknown metabolic roles, YNL089 and YDR267, with unknown metabolic role have been identified as probable candidates to encode for ED pathway enzymes [27]. The second route is the pentose phosphate pathway, which provides additional carbon influx into central metabolism and is essential for pentose metabolism. This pathway is also an important source of NADPH essential for biomass synthesis. Pentose sugars such as xylose are metabolized by E. coli by isomerization to xylulose and phosphorylated to xyloluse-5-phosphate and enter the pentose phosphate pathway [28]. Xylose-fermenting yeasts, convert xylose to xylulose in a two step process by xylose reductase and xylitol dehydrogenase [29]. However, S. cerevisiae cannot ferment xylose, unless supplemented with NADPH-dependent xylose reductase and NADdependent xylitol dehydrogenase. The introduction of these two pathways in S. cerevisiae results in a net consumption of NADPH and NADH production. The physiological implications of this will be discussed subsequently.

TCA cycle and anaplerotic reactions

The end product of glycolysis is pyruvate, which can be fermented to organic acids and ethanol, or be metabolized in the TCA cycle to generate energy and biosynthetic precursors. The TCA cycle is important from a redox standpoint since it generates four moles of NADH and one mole of FADH₂ for every mole of pyruvate according to the net reaction:

 $Pyruvate + GDP + FAD + 4NAD + P_i \rightarrow 3CO_2 + GTP + FADH_2 + 4NADH$ (5)

In case of E. coli, entry of pyruvate into the TCA cycle is fairly simple, since the TCA cycle enzymes present in the cytoplasm. However, in S. cerevisiae the TCA cycle is restricted to the mitochondria and therefore pyruvate must be transported across the mitochondrial membrane to fuel the TCA cycle. This transport is carried out by a carrier protein, YIL006 [30]. Upon entry into the mitochondria pyruvate is oxidized to acetyl CoA by the pyruvate dehydrogenase enzyme complex, encoded by aceE, aceF and lpd in E. coli and PDA1, PDA2, LAT1 and LPD1 in S. cerevisiae (Table 5.1). Citrate synthase (encoded by gltA in E. coli and CIT1 and CIT2 in S. *cerevisiae*) ligates acetyl CoA with oxaloacetate and forms citrate. Isocitrate dehydrogenase (encoded by *IDH1* and *IDH2* in *S. cerevisiae* and *icd* in *E. coli*) in yeast uses NAD as the cofactor in oxidizing isocitrate to 2-keto glutarate [31], while E. coli used NADP as the cofactor for this conversion [32]. This difference likely impacts the availability of NADPH for biomass synthesis and maintaining a redox balance. The final step in the TCA cycle (conversion of malate to oxaloacetate) is mediated by malate dehydrogenase (mdh gene in E. coli and MDH2 in S. cerevisiae) and generates NADH in both the organisms. However, in E. coli this conversion could also be carried out by a membrane bound enzyme that uses quinol as the co-substrate and is expressed by the *mgo* gene. This gene is reported to be very active during exponential growth [33].

Another role of the TCA cycle is to generate biosynthetic precursors from intermediates such as oxaloacetate, 2-keto glutarate and succinyl CoA. As carbon is drained from these intermediates, the flux in the TCA cycle would decrease if no additional influx of carbon occurs. Anaplerotic reactions serve this purpose of replenishing carbon in the TCA cycle. The PEP carboxylase (encoded by *ppc*) is the main anaplerotic reaction that forms oxaloacetate from PEP in *E. coli*

[34,35]. In *S. cerevisiae* pyruvate carboxylase (encoded by *PYC1* and *PYC2*) supplies oxaloacetate from pyruvate [36]. The reason these two organisms have evolved different anaplerotic mechanisms is unclear, but the *E. coli ppc* gene can complement the anaplerotic function in PYCA *S. cerevisiae* [37] and the *pyc* gene (from *Rhizobium etli*) can restore the metabolic defects brought about by *ppc* mutation in *E. coli* [38].

An important observation after comparing the genetics of central metabolism in these two organisms is that *S. cerevisiae* more often has multiple genes perform the same function under a variety of conditions, while *E. coli* has a single gene which exhibit greater flexibility. The redundancy of gene functionality in *S. cerevisiae* is likely due to duplication either along with the genome or independent of it [39,40]. The importance of multiple genes whose products provide the same function (such as isozymes, for example) and their role in providing a back-up function in eukaryotes is to maintain network robustness [41,42,43], while prokaryotes do not have this mechanism. The evolutionary driving force in retaining these paralogs is not network robustness, but rather the specialization of the duplicate genes in function, expression and localization [40], and many of the genes mentioned likely played a key role in the evolution of *S. cerevisiae* to possess a respiro-fermentative metabolism [44]. The evolution of the *S. cerevisiae* genome provides further information on the differential regulation of isozymes [42] and why they are not co-expressed [41].

Redox Homeostasis

Redox homeostasis in cells is a characteristic function of their physiological state. A large shift in redox induces a change in the physiological state of the cell. The parts of the metabolic

network that are most susceptible to shift in redox, of course, are those which involve the redox cofactors. The high connectivity of the redox cofactors (NAD(P) and NAD(P)H) within the metabolic networks of E. coli and S. cerevisiae illustrate the potential impact of a change in these cofactors on metabolism [45]. The genome scale models for E. coli [46] and S. cerevisiae [47] account respectively for 71 and 65 reactions that involve NADH and 49 and 78 reactions that involve NADPH. In both organisms most reactions involving NADH belong to carbohydrate catabolism. NADH is also involved in the synthesis of amino acids from intermediates of central carbon metabolism (Figure 5.1). On the other hand, NADPH is involved primarily in the biosynthesis of building blocks of the cell, particularly amino acids and lipids. The stoichiometric biomass composition of a yeast cell is CH_{1.82}N_{0.16}O_{0.58} and of an *E. coli* cell is CH_{1.94}N_{0.25}O_{0.52}P_{0.025} [48], both of which are slightly more reduced (degree of reduction per carbon atom is 4.18 and 4.28, respectively) than glucose (degree of reduction per carbon atom 4.0). The increase in the degree of reduction for biomass compared to glucose is a result of the consumption of reducing equivalents in the form of NADPH during the assimilation of glucose and ammonia [49,50]. The decrease in the cellular concentration of NADPH due to biomass synthesis is partly compensated by the generation of NADH to maintain redox balance. The loss of carbon from glucose as CO₂ (zero degree of reduction) in the pentose phosphate pathway and in the conversion of pyruvate to acetyl CoA implies that there is excess NADH generated. Although the exact amount of assimilatory NADH production depends on the growth conditions and biomass composition, the formation of 1 g of yeast biomass from glucose and ammonia is accompanied by the net synthesis of 10 mmol of NADH [51].

NADH is oxidized back to NAD by two NADH dehydrogenases (NDH-I and NDH-II) in *E. coli*. Although the functionality of these two enzymes appears redundant, a closer look reveals distinct tasks. The proton pumping NDH-I of *E. coli* is encoded by the *nuoA-N* genes and is considered the prokaryotic version of the mitochondrial Complex I of the eukaryotic respiratory chain [52]. NDH-I is structurally complex with 14 subunits, and the functions of most are still not clear.

Figure 0-1

The functional distribution of the metabolic reactions involving the redox cofactors (NADH and NADPH) in the genome-scale models of *E. coli* [46] and *S. cerevisiae* [47]. The pie-charts A and C represent the reaction distribution in *E. coli* and the pie-charts B and D represent the reaction distribution in *S. cerevisiae*. A majority of the reactions that involve NADH (A and C) belong to the central metabolism, while those that involve NADPH are used in the synthesis of biomass precursors. The functional categories of *E. coli* are based on Riley classification [117] and those of *S. cerevisiae* are from MIPS.



In contrast NDH-II is a single subunit enzyme of 47 kDa with no transmembrane elements that readily oxidizes NADH [53]. This second NADH dehydrogenase is encoded by the *ndh* gene, and is severely repressed by the FNR global regulator under anaerobic and microaerobic conditions [54]. *E. coli* therefore appears to predominantly rely on NDH-I for NADH oxidation under these conditions and during the transition into the stationary phase [55]. Unlike the primary NDH-I, NDH-II is not coupled to proton translocation nor is affected by the common inhibitors of mitochondrial complex I respiration such as rotenone. NDH-II homologs or analogs are widespread in bacteria, providing an alternate respiratory pathway for NADH oxidation that is not coupled to proton translocation for NADH oxidation that is not coupled to proton translocation for NADH oxidation that is not coupled to proton translocation for NADH oxidation that is not coupled to proton translocation for NADH oxidation that is not coupled to proton translocation for NADH oxidation that is not coupled to proton translocation [56]. A version of NDH-II has also been found in *S. cerevisiae* [57].

In contrast to *E. coli*, redox homeostasis in *S. cerevisiae* is compartmentalized [58], and requires separate mechanisms to maintain redox in the cytosol and in the mitochondria. Although recently proteins responsible for the transport of NAD across the mitochondrial membrane have been discovered [59], there is no evidence of an NADH carrier. Glycolysis is the principal source of cytosolic NADH, which is oxidized by two NADH dehydrogenases (encoded by *NDE1* and *NDE2*) bound to the external mitochondrial membrane with their catalytic site facing the cytosol [60]. Mutants lacking both these genes are severely impaired in their respiratory capacity, but still exhibited oxidative growth at very low rates of glucose uptake [61]. The rate of NADH oxidation reduced three-fold in the *NDE1 MDE2* double mutant, indicating the relative importance of these genes in respiration [60]. These two genes are believed to be the

result of genome duplication [43], and have developed distinct functions to justify their retention in the genome [40]. Moreover, although *NDE1* and *NDE2* share the same function of cytosolic NADH oxidation, they differ in regulating oxygen consumption flux [62]. It was recently concluded that NDE1 has higher priority in transferring electrons to the respiratory chain [62]. In addition to the two NADH dehydrogenases, the glycerol-3-phosphate shuttle also plays a key role in maintaining cytosolic redox balance [63]. This shuttle system is located on the inner membrane of the mitochondria with the catalytic site facing the cytosol. This system oxidizes NADH to NAD by the cytosolic glycerol-3-phosphate dehydrogenase while reducing dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate [63].

In order to oxidize mitochondrial NADH, *S. cerevisiae* also has the internal NADH dehydrogenase (encoded by the *NDII* gene), which is also bound to the mitochondrial membrane with its catalytic site facing the mitochondria [57]. This is the only mechanism for the oxidation of mitochondrial NADH since the mitochondria isolated from *NDI1* mutants cannot oxidize substrates that generate NADH [57]. The NDI1 protein contains a noncovalently-bound FAD as the sole prosthetic group [57] and shares functionality with similar rotenone-insensitive NADH dehydrogenases from many other organisms, including *E. coli* and humans (Figure 5.2). All these proteins have an evolutionarily conserved $GXG(X)_2G$ consensus motif in a $\beta\alpha\beta$ -fold [64]. The primary structure of these proteins also reveals a conserved glutamate residue (Figure 5.2), which is believed to be involved in the binding of the flavin cofactor. The similarity in sequence and function between the *NDI1* gene from *S. cerevisiae* and the AMID from humans opens the possibility of using this gene as a potential therapy for many of the respiratory diseases in humans [65,66,67].

Figure 0-2

Alignment of the amino acid sequences of the non-proton translocating NADH dehydrogenases from *E. coli* (V00306), *S. cerevisiae* (X61590), *A. vinelandii* (AF346487), *S. tuberosum* (AJ245861), *H. sapiens* (NP_116186) and *N. crassa* (AJ236906). Among all these species, there is a highly conserved GXGXXG motif with a $\beta\alpha\beta$ -fold with a covalently bound flavin. The structural and functional similarity of the NADH dehydrogenases with the human gene opens the possibility of replacing the defective human gene with one of these homologs.

E.coli_NDH S.cerevisiae_NDI1 A.vinelandii_ndh S.tuberosum_nda1 H.sapiens_ANID N.crassa_ndh64	110 I YSNKRLTTSTNTTV SITPLASPLLTQFI SKATNQGDITVPKL	120 I I RFASTRSTGU .QFTKQYSTN- ALNPRRGGPF	130 II. ENSGAGPTSFI DI NLPILEIFLDI	140 MT KTMKV IDP QH MT IVVGLEATKS MGS QV SVE DD SEEKKKI	150 TPLKKIVIVG SGALHVVIVG SGALHVVIVG SGALHVVIVG SGALHVVIVG	160 GGAGGLEMAT SGWGAISFLK GGAGGVELAT SGWAGCRLMK GGFGGTAAAS GGWGSVALLK	170 i i . QL GHKLG HID TKKY RL GKTMG D ID TNIY QL QALNY ELNPDDY
	218	228	230	248	258	260	270
		1 1	1		1	1	1 1 -
E.coli NDH	LDEGVDALSYLA	HARNHGFQ	FQLGSVIDID	EAKTITIAE	LRDEKCELLV	PERK	IAYD
S.cerevisiae NDI1	VDEKSI IEP IVNFA	TKKKGNVT	YYEAEATSINE	PDRNTVTMKS	LSAVSOTYOP	ENHTGTHQAE	PAEIKYD
A.vinelandii ndh	LNSTGDELNYVA	OAKWNNFE	FOYGRMCGLDE	ANKRIRLAA	OPAOEDRAPL	PERE	LEYD
S. tuberosum pdal	LEEDSWAEPTORTO	PAWSTOPASY	FFI ANCHATDE	DUMMTECET	WTEGNETI EA	WKFN	WSVD
H saniens AMTD	VETGEAKKTETSYS	WTEKDN	FROGINVETDI	KNOMMU LOG	GE		ALPES
W. crassa ndb64	LEUNSIVEPTRATI	DBURGH	VIDAAAEDUDE	SSBLVEVSO	KDDDCNE	VDFV	WPVD
A.CIUSSU_MUNO4	ELEASEVEL IRALI	DRVRom	1 INTERVE	. 33 111 7 11 7 3 9		vin 1	VI 10
	310	320	330	340	350	360	370
E.coli NDH	OARRFHOEMLNLFL	KYSANI	GANGKVNIA IV	GGGATGVEL	SAELHNAVKO	LHSYGYKGLT	NEALNVT
S.cerevisiae NDI1	NSLEMBRIFAANTE	KANLLPKGDP	ERRRTTSIVV	GGGPTGVEA	AGETODYVHO	DTR-KFTPAL	AEEVOIH
A.vinelandii ndh	0AERFRRPLLSHVI	RAHASN	DDGHOVKVA TV	GAGAT GVEL	AAELRHASKE	LVAYGLER TP	PENLSIT
S.tuberosum pdat	HAOETBEKILLNIM	I.SDVP GVSEF	EKRBLLHCVVV	GGGPTGVEF	SCELSDETLK	DVHORYAHVK	-DYTHVT
H.saniens AMID	AATOAVEDMUR		OVORSPETION	ICCCS ACVES	AAETKT	EVPERENTLT	A LAWBER
W GWAGGA DAb64	DEDATDNETTONT F	I COLDETCOR	FONDI I CERNI	CCCDTCVE	ADDIEDI NE	DI TI NEDDI I	DUPTCUL
n.vrassa nullo4	NURATENTIA	LOVER 1190E	EDDDLL OF VVV	HOODE T ON PL	OUPPE APPE	VLIDIE PROL	NINE LOVI

The reader is directed to more detailed discussion on the different paths of NADH oxidation, including mitochondrial redox shuttles in *S. cerevisiae*, and their implications on metabolism in excellent reviews by Bakker et al. [68], and Rigoulet et al. [69].

Electron transport and bioenergetics

The NADH dehydrogenases described in the previous section are a part of a highly evolved, regulated cascade of reactions involving multi-subunit enzyme complexes, the ETC. In prokaryotes and eukaryotes, electrons are transferred from NADH to oxygen through a cascade of large enzyme complexes: NADH:ubiquinone oxidoreductase or NADH dehydrogenase (Complex-I), succinate dehydrogenase complex (Complex II), ubiquinol:ferricytochrome C oxidoreductase or cytochrome bc₁ (Complex III) and ferrocytochrome C oxidoreductase or cytochrome oxidase (Complex IV). These enzyme complexes are usually coupled to proton translocation (Figure 5.3). Complex I is the largest and the most complex with the mammalian enzyme consisting of 43 subunits in an unknown stoichiometry [70], and it is still not clear how proton translocation is coupled to electron transfer in Complex I. The succinate:ubiquinone reductase (Complex II), is a component of the TCA cycle and participates in the electron transport chain by transferring electrons to the ubiquinone pool by oxidizing succinate to fumarate. Complex II is anchored to the membrane by a b-type cytochrome and does not translocate protons, and therefore it only feeds electrons to the electron transport chain [71]. Cytochrome bc₁ (Complex III), the best understood of the respiratory enzymes, delivers electrons from ubiquinol to cytochrome c. It couples this redox reaction to the generation of a proton gradient across the membrane [72].

Figure 0-3

The series of reactions involved in the transport of electrons from either NADH or succinate to a common quinone pool and then subsequently to oxygen, where it is reduced to water. The transfer of electrons is catalyzed by Complex I (NADH dehydrogenase), Complex II (Succinate dehydrogenase), Complex III (Cytochrome bcl) and Complex IV (Cytochrome oxidase). Except

for the Complex II, the remaining enzyme complexes couple electron transport with proton translocation to generate a proton gradient across the membrane. The ATP synthase (Complex V) captures this gradient in the form of ATP. The thick lines indicate proton translocation and the fine line follows the path of electrons from the donors (NADH or succinate) to oxygen through cytochrome *c*. Most eukaryotes (with the notable exception of *S. cerevisiae* and *S. pombe*) also possess a cyanide-resistant alternative respiratory pathway (AOX). *S. cerevisiae* also lacks the Complex I in the respiratory chain.



Cytochrome oxidase (Complex IV) also generates a transmembrane proton gradient, but by a different mechanism than cytochrome bc_1 . Its substrate, cytochrome c, is a water-soluble hemoprotein that donates electrons on the cytoplasmic side of the mitochondrial inner membrane. These electrons are transferred to the active site, which contains a heme iron and a copper, and they are used to reduce O_2 into two water molecules [73].

The resulting proton gradient across the membrane is proportional to the free energy of electron transfer reactions. In the presence of ADP, protons flow down the gradient back into the mitochondrial matrix (in eukaryotes) or into the cytoplasm (in prokaryotes) and is facilitated by ATP synthase, which captures this energy and stores it as ATP. The fundamental role of this highly exergonic reaction cascade is to transfer electrons from the electron donors, such as NADH, formate or lactate to electron acceptors such as oxygen, nitrate, nitrite, etc, using quinones (ubiquinone, menaquinone or dimethylmenaquinone) as intermediates (Table 5.2). NADH is the preferred currency of redox metabolism in sugar catabolism since the NAD/NADH redox couple has the highest reducing power (-320 mV) compared with other common aerobic redox couples such as FAD/FADH₂ (-220 mV). Therefore, cells prefer to use NADH as the electron donor in most catabolic reactions. Among the various electron acceptors shown in Table 5.2, oxygen has the highest oxidizing power, with the potential of the $\frac{1}{2}O_2/H_2$ couple being 820 mV. Therefore, in the transfer of electrons from NADH to oxygen, for example, there is a net potential difference of 1.14 V, corresponding to a free energy of -220 kJ/mol. Since the redox potential is linearly related to the Gibbs free energy of a reaction, the large redox potential involved in the transfer of electrons from NADH to oxygen reflects the large energy generated in aerobic processes.

Table 0-2

List of common electron donors and acceptors that contribute to the ETC in *E. coli* and *S. cerevisiae*. The list was compiled using the EcoSal database (http://www.ecosal.org), EcoCyc database (http://www.ecocyc.org) and Saccharomyces Genome Database (http://www.yeastgenome.org). Electrons travel through the dehydrogenases from the donor substrates to acceptors using the common quinone pool as an intermediate step.

N.I.: not identified

	Dehyd	rogenases	Rec	luctases	Theatree A conter
Electron Donor	E. coli genes	S. cerevisiae genes	E. coli genes	S. cerevisiae genes	Electron Acceptor
Formate	fdnGHI fdoGHI	FDH1 FDH2 VPI 276W	cyslJ	MET10, ECM17	Sulfite
			frdABCD	FRDS, OSM1	Fumarate
Hydrogen	hyaABC hybABC	N.I.	torACD	N.I.	TMAO
NADH	nuoA-N ndh	NDE1, NDE2 NDI1	dmsABC	N.I.	DMSO
			nrfAB	MET8	Nitrite
l actate	IIdD	CYB2			
	dld	DLD1, DLD2, DLD3	narGHI		Nitroto
	glpD	GPD1, GUT2	narzrv napABC	N.I.	INITIALE
GIACEI OI-3F	glpABC	GPD2			
Succinate	sdhABCD	SDH1, SDH2, SDH3. SDH4	cydAB	COX12, COX13	Oxygen
-	ſ		cyxAB		
Pyruvate	рохВ	N.I.			

There are substrate-specific dehydrogenases that are responsible for the preliminary transport of electrons from the substrate (Table 5.2). As described earlier, in E. coli the NDH-I is the primary proton pump and results in translocating two protons (per electron) whereas NDH-II does not pump protons. These two enzymes initiate the transfer of electrons from NADH to the quinone pool. NDH-I assumes the role of Complex I in E. coli, but the presence of NDH-II to perform the same function results in a branched respiratory pathway. Under aerobic conditions, oxygen is the electron acceptor of choice and there are two cytochrome oxidases (cytochrome bd and cytochrome *bo3*) that carry the electrons from the quinone pool ultimately to oxygen. While cytochrome bo3 (prokaryotic Complex III) pumps 2 protons per electron transported, cytochrome bd oxidase (prokaryotic Complex IV) pumps only one proton. Therefore, the number of protons pumped into the periplasm could vary between 1 and 4 per electron transported in the aerobic oxidation of NADH in E. coli (Figure 5.4), offering it great flexibility in generating a proton gradient from NADH oxidation. The electron flux through these enzymes will depend on the enzyme levels, NADH availability, quinone levels as well as the kinetics of the involved enzymes. The different bioenergetic efficiency of the branched electron transport chain in E. coli has been experimentally demonstrated by comparing growth in steady-state cultures of strains that are engineered in different components of the respiratory pathway [74]. Therefore, E. coli can utilize various combinations of the respiratory chain resulting in a large variation of the bioenergetic efficiency, implying that the combination that results in maximum energy need not be the optimal path for electron flux. It is widely believed that this optimal path of electron transport and resulting energetic efficiency depends on the cellular redox state and the need to regenerate NAD from NADH.

Figure 0-4

A schematic illustration of the protons generated per electron transported in *E. coli* (A) and in *S. cerevisiae* (B). The branched respiratory chain in *E. coli* permits greater flexibility in the generation of energy (proton gradient) per NADH oxidized. While the number of protons generated (per electron transported) in *E. coli* could vary between 1 - 4, *S. cerevisiae* could either translocate either 1 or 2 protons, depending on which cytochrome oxidase it uses. The electron pathways in *E. coli* (A) were redrawn from Calhoun et al. [74].



Unlike many organisms including other yeasts, *S. cerevisiae* lacks a multi-subunit NADH dehydrogenase that functions as the Complex I [75] to couple the oxidation of mitochondrial NADH to respiration (Figure 5.3). This deficiency is rectified partially by the *NDI1* gene, which encodes for the non-proton pumping, rotenone-insensitive, single subunit internal mitochondrial NADH dehydrogenase. The external NADH dehydrogenases (NDE1 and NDE2) also are non-proton translocating with NDE1 being the primary enzyme for cytosolic NADH oxidation under respiring conditions [76]. Moreover similar to the complex II from other organisms, the succinate dehydrogenase complex (Complex II) is not capable of pumping protons [71], which leaves Complex III (cytochrome bc1 oxidase) and Complex IV (cytochrome *c* oxidase) to generate a proton gradient across the mitochondrial membrane in *S. cereviasiae*. Cytochrome *bc1* oxidase is composed of 10 subunits [77] and pumps 2 protons per electron transported [72]. Cytochrome C oxidase (Complex IV) is the terminal enzyme in the electron transport chain and consists of 13 subunits in *S. cerevisiae* [78] and pumps only 1 proton per electron transported [73].

Unlike the structure of the respiratory chain in *E. coli*, respiration in *S. cerevisiae* is not branched since none of the NADH dehydrogenases (NDE1, NDE2 or NDI1) pump protons. Therefore there can be very little variation in the proton gradient that the *S. cerevisiae* respiratory chain can generate as there can only be either 1 or 2 protons (as opposed to 1 - 4 protons in *E. coli*) that can be pumped per electron transported, depending on whether they are transported by Complex IV or Complex III, respectively (Figure 5.4). The proton gradient that is generated is converted to energy by the ATP synthase, which is sometimes called Complex V. The difference in the efficiency of the respiratory chain (amount of proton gradient generated per mole of NADH

oxidized) between *E. coli* and *S. cerevisiae* is directly related to the amount of energy (ATP) that is generated. This could be quantified by number of moles of ATP generated per mole of atomic oxygen used in oxidative phosphorylation, known as the P/O ratio. The typical value of the P/O ratio in *E. coli* is in the range of 1.5 - 2.0 (calculated from the data in Russell and Cook, 1995 [79]), while it is believed to be closer to 1 in *S. cerevisiae* [51]. The lower P/O value for *S. cerevisiae* is due to the oxidation of substantial amounts of NADH by the NADH dehydrogenases without generating a proton gradient. This also explains why other yeasts with proton-pumping Complex I have more efficient respiratory metabolism [80].

Engineering redox balance and impact on overflow metabolism

The first generation of metabolic engineering applications involving the overexpression of the rate-limiting steps in product formation and/or deletion of pathways leading to byproduct formation has applications only in pathways that are decoupled with regulation. Since a majority of the pathways are controlled by regulatory mechanisms, the focus of metabolic engineering is now turning towards engineering global regulators, which affect large parts of metabolism [81]. Global regulators are frequently sensitive to cellular redox and energy levels and therefore, engineering the levels of NADH or ATP is emerging as a powerful metabolic engineering tool.

During stress, when the cells need rapid energy generation, they consume glucose rapidly to generate ATP. When the rate of glucose consumption exceeds the cellular capacity for its complete oxidation, alternate products other than just biomass and CO_2 are formed to prevent its intracellular accumulation. This phenomenon where surplus glucose is converted to other products is referred to as overflow metabolism, a mechanism that is very prevalent in most

facultative anaerobes including *E. coli* and *S. cerevisiae*. In *E. coli* acetate is the overflow metabolite while ethanol is considered to be the product of overflow metabolism in *S. cerevisiae*. Besides the obvious disadvantages of inefficient yields during aerobic growth, overflow metabolites have also been reported to hinder recombinant gene expression and growth in both the organisms [82,83]. Rapid glucose metabolism results in a high NADH generation rate (see equations 4 and 5), which needs to be oxidized also at a sufficiently high rate to maintain redox balance. A saturation in the respiratory capacity limits the rate of NADH oxidation, and the rate at which NADH is produced exceeds its consumption causing an increase in the intracellular level of this cofactor [84,85]. Moreover, other factors such as inhibition of the enzymes of the TCA cycle by NADH and glucose repression are also believed to contribute to overflow metabolism.

A substantial portion of literature on the regulation of sugar uptake in yeasts is devoted to overflow metabolism. There are different regulatory mechanisms that govern the metabolism of sugars that affect the balance between respiration and fermentation. While these regulatory effects were studied in the context of sugar metabolism specifically by yeasts, we present evidence if they are also prevalent in *E. coli* as well. These mechanisms named after the scientists that discovered this phenomenon are summarized in Table 5.3. The Crabtree effect is the most widely observed regulatory phenomenon on sugar metabolism. It is characterized by the appearance of fermentation products during high glucose uptake rates and is believed to be caused by the saturation of the respiratory pathways. This is also known as the long-term Crabtree effect [4].

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Regulatory phenomena governing carbohydrate uptake and overflow metabolism.

Regulatory phenonemon	Effect	Cause	S. cerevisiae	E. coli	Comments	References
lbtree Effect	Appearance of fermentation products under high growth rates (glucose uptake rates)	Insufficient capacity of NADH oxidation due to saturated respiratory pathways	Yes	Yes	This phenomenon was first observed in tumor cells , and was subsequently observed in S. cerevisiae and E. coli	
lyver effect	Inability to ferment di- and oligosaccharides anaerobically	Suppression of sugar carrier causing slower sugar uptake anaerobically	N	No		
sters effect	Faster conversion of glucose to ethanol under aerobic conditions compared with anaerobic conditions	Imbalance in redox, resulting in high NADH/NAD ratio	No	No		
steur effect	Faster utilization of glucose in the absence of oxygen, than in its presence and suppression of ethanol fermentation under aerobic conditions	Higher kinetic affinity of respiratory pathways for pyruvate, NADH, etc	Yes	No	This effect is seen in S. <i>cerevisiae</i> only at low growth rates	

The instantaneous occurrence of aerobic fermentation when cells are subjected to excess glucose is known as the short-term Crabtree effect [86], which is also due to insufficient respiratory capacity. Kluyver effect [87] is the ability of cells to metabolize oligosaccharides only under aerobic conditions. This apparent dependence of sugar utilization on the respiration is believed to be caused by the inability to synthesize components of respiration [88]. Neither E. coli nor S. cerevisiae exhibit this phenomenon. Custers effect [89] refers to faster conversion of glucose to ethanol under aerobic conditions than anaerobically. This is caused by the generation of acetate by NAD-dependent aldehyde dehydrogenase leading to high NADH:NAD ratio, which inhibits glycolysis under anaerobic conditions [90]. This effect is also not observed in E. coli nor S. cerevisiae. The Pasteur effect [91], in contrast to the Custers effect, refers to the faster glucose utilization under anaerobic conditions and even suppression of fermentation in the presence of oxygen. Although the exact cause of this phenomenon is still under debate, the higher kinetic affinity of the respiratory pathways for pyruvate and NADH than the fermentative pathways is believed to play a key role [92]. This phenomenon is present in S. cerevisiae only at low growth rates, and absent in E. coli.

All the regulatory mechanisms controlling glucose uptake and overflow metabolism are directly or indirectly related to redox balancing. Therefore, manipulating redox cofactors is emerging to be an attractive approach to understand overflow metabolism better as well as a means to eliminate it. Recent examples of perturbing the levels of NADH in *E. coli* include overexpressing heterologous NAD-dependent formate dehydrogenase, which converts formate to CO_2 with the generation of equimolar NADH [93,94,95]. This perturbation generated typical anaerobic products such as ethanol, lactate and acetate, even under fully aerobic conditions [94]. Under anaerobic conditions, increasing NADH availability favored the formation of more reduced products, as indicated by higher ethanol:acetate ratio [93]. These metabolic changes were brought about by an apparent increase in the NADH/NAD ratio (defined as the intracellular redox ratio) that simulated increased anaerobicity, causing the generation of organic acids and ethanol under aerobic conditions or increasing the formation of more reduced products under anaerobic conditions [93,94].

In contrast, overexpressing water-forming NADH oxidase in E. coli increased the utilization of NADH in a futile cycle and decreased the intracellular redox ratio [96]. This manipulation created a more "aerobic" environment that enabled E. coli to consume glucose faster and respire faster, as indicated by the increase in specific CO₂ evolution rate [96]. The impact of this perturbation was more prominent at rapid glucose consumption rates, when the generation of NADH is presumably higher. Increased oxidation of NADH also reduced acetate generation and reduced biomass yield, since the portion of NADH that would have been utilized in the ETC is oxidized to NAD by non-proton translocating NADH oxidase. Although acetate formation is known to occur at a growth rate above of about 0.4 h⁻¹ in wild-type *E. coli* for over 30 years [1], the molecular mechanisms and the genetic triggers have very recently been identified [96]. Upon correlating the physiological profile of *E. coli* from different growth rates with intracellular redox ratio, defined as [NADH]/[NAD], it was observed that the commencement of acetate generation occurred when this redox ratio reached a value of 0.06 [97]. This ratio remained unchanged even in a mutant strain with increased NADH oxidation activity, although there were several other significant differences observed. It was also observed in the same study that intracellular level of pyruvate increased just prior to the onset of acetate overflow in both the

strains, although this increase was slightly moderated in the strain with enhanced NADH oxidation. These observations strongly suggest a key role for intracellular redox and pyruvate levels in aerobic acetate generation. By associating the transcription profile at different growth rates with these observations, it was discovered that several of the key central metabolic genes from the TCA cycle and respiration were repressed upon acetate formation after exhibiting an initial increase in their expression profiles [96]. Analysis of promoter regions upstream of the genes that were correlated with the redox ratio revealed significant enrichment of a consensus sequence that was identified to be the binding site for the dual transcriptional regulator, ArcA (Aerobic Respiratory Control). This protein belongs to the two-component signal transduction system that controls global gene regulation together with the ArcB protein [98,99]. The regulation of the genes that are sensitive to the redox ratio in *E. coli* are consistent with previous observations that the Arc system is triggered by intracellular redox levels [100]. An important outcome of this study was the discovery that at high growth rates (corresponding to rapid glucose uptake), the retardation of the TCA cycle is primarily due to repression of the genes and not due to inhibition of the enzymes, as was previously believed for a long time [3].

Despite the well-documented evidence of the repression of TCA cycle genes and respiration genes by the ArcAB system, its relevance in governing overflow metabolism was overlooked. Based on these observations, the following hypothesis was proposed for the mechanism of acetate formation in *E. coli* [96]. The high values of [NADH]/[NAD] prevalent at high growth rates signal the activation of the ArcAB two component system, which is characterized by the phosphorylation of ArcB. The activated form of this protein triggers the DNA-binding action of its response regulator, ArcA, and thereby repressing the transcription.

Figure 0-5

The proposed model for the regulation of aerobic acetate overflow in *E. coli* [96]. According to this model, when the redox ratio exceeds a certain threshold, it signals the activation of the Arc dual component system. The sensor kinase (ArcB) is phosphorylated and activates its cognate response regulator (ArcA), which binds to specific consensus motifs on the DNA. Since several of the central metabolic genes, including those in the TCA cycle and respiration have this motif upstream of their ATG-start codon, their transcription is repressed, thereby preventing further increase of the redox ratio.



Since several of the key genes that produce NADH contain ArcA binding sites, these genes are repressed to cut down further generation of NADH (Figure 5.5). ArcA also represses the cytochrome oxidases *bd* and *bo3* [101], which are involved in oxidative phosphorylation, to avoid the wasteful expression of these complexes. This simple, but elegant, mechanism of transcriptional regulation to moderate intracellular NADH levels is further elucidated by evaluating the performance of the *arcA*-deletion mutants. The metabolism in these strains is characterized by an upregulation in most of the TCA cycle genes, concomitant with higher biomass yields and increased threshold for glucose uptake before the manifestation of overflow metabolism [96]. The relief in the repression on these genes increased the capacity of the TCA cycle as well as respiration and consequently, resulted in lower acetate generation (Figure 5.5).

Unlike in *E. coli*, redox metabolism in *S. cerevisiae* is characterized by the absence of a transhydrogenase activity to catalyze the conversion of NAD and NADPH to NADP and NADH [102,103] and the compartmental restriction of NADH reoxidation, since the mitochondrial inner membrane is not permissible to NADH [58]. Introducing a transhydrogenase reaction from *E. coli* in *S. cerevisiae* resulted in increased glycerol and 2-oxoglutarate generation under anaerobic conditions, indicating increased NADPH consumption (and NADH generation) [104]. Since a majority of the NADPH is consumed for biomass synthesis by the NADPH-dependent glutamate dehydrogenase (encoded by GDH1) [105], deleting this gene is one logical way to reduce the demand for NADPH. However, the central role of glutamate dehydrogenase reaction in ammonia assimilation requires its presence for amino acid metabolism. Therefore, this gene was functionally replaced with its NADH-dependent isozyme (encoded by GDH2) or with the GS-GOGAT pathway catalyzed by ATP-dependent glutamine synthase (encoded by GLNI) and

NADH-dependent glutamate synthase (encoded by GLTI) [106]. These strategies decreased the demand for NADPH either by the direct replacement of the NADPH-dependent reaction with its NAD-dependent counterpart (in former case), or by substituting NADPH consumption with combined ATP and NADH consumption (in latter case). While either of these two strategies to decrease the demand for NADPH did not have any affect on the yield of ethanol and glycerol, they reduced the critical dilution rate [106]. In order to meet the high cellular demand for NADPH for biomass synthesis, cytosolic pyruvate carboxylase (encoded by PYC2) and malic enzyme (MAE1) were overexpressed together [107]. The net effect of this manipulation also results in the production of NADPH at the expense of NADH and ATP. Similar to the NADPH perturbations described above, this strategy of increasing NADPH availability did not alter ethanol or glycerol production. Therefore, it appears that NADPH has more relevance in biomass synthesis and a relatively minor role in controlling overflow metabolism.

Ethanol as a product of overflow metabolism has a fundamental difference from acetate. Unlike acetate, ethanol is redox neutral with glucose. Therefore, aerobic ethanol formation cannot account for a limitation in the NADH oxidation capacity in *S. cerevisiae*. Nevertheless, similar to the respiro-fermentative metabolism of *E. coli*, yeast produces ethanol as a result of overflow metabolism, above a growth rate of about 0.3 h^{-1} [108]. Therefore using a similar strategy of increasing NADH oxidation by overexpressing bacterial NADH oxidase in *S. cerevisiae* did not significantly alter ethanol, but reduced glycerol generation [109]. During respiratory growth, the NADH produced is reoxidized by the normal respiratory processes to meet cellular energy demands. The problem of excess NADH that cannot be oxidized by respiration is solved by the formation of glycerol, which consumes equimolar quantities of NADH. It is now widely

accepted that aerobic glycerol formation by *S. cerevisiae* is a consequence of an imbalance in cytosolic redox [68,69] and can therefore be considered overflow metabolism, according to the definition. The overexpression of bacterial NADH oxidase consumed the excess NADH that would otherwise have been oxidized by glycerol synthesis. Therefore, in *S. cerevisiae* this bacterial enzyme is localized and functional in the cytoplasm. This is a reasonable inference since bacterial proteins lack the leader peptide sequence to direct mitochondrial localization. Moreover, bacterial NADH oxidase could also restore growth and reduce glycerol accumulation in *NDE1* Δ *NDE2* Δ *GUT2* Δ mutant [109].

Electrons from cytosolic NADH are transferred to the ETC, which is located in the mitochondrion. Therefore, high levels of cytosolic NADH could decrease the TCA cycle activity by saturating the capacity of electron transport in the ETC. Under such conditions the TCA cycle splits into two branches and ceases to operate as a complete cycle and when cytosolic NADH level is decreased, the two branches function together as a cycle again [110]. Previous results demonstrated the dominance of Nde1 in transporting electrons from cytosolic NADH to the ETC [62], so this protein plays a key role in regulating mitochondrial oxidative metabolism. Under physiological conditions, the kinetic constraints for electron transfer in the ETC should lead to a higher redox potential in mitochondria than in the cytosol. When functioning as a cycle, the TCA cycle generates surplus NADH. Although the NDI1 protein specifically oxidizes mitochondrial NADH, it is repressed by glucose [24] and therefore functions sub-optimally during rapid glucose uptake. This could lead to insufficient oxidizing power in the mitochondria that could lead to accumulation of NADH, inducing the retardation of the TCA cycle. The high glycolytic flux has then to be shunted to ethanol formation in order to oxidize the NADH

generated in glycolysis. Therefore, aerobic ethanol formation in appears to be directly related to the accumulation of mitochondrial NADH. This hypothesis was validated by expressing the cyanide-resistant alternate respiratory pathway by cloning the AOX gene (encoding for alternate oxidase) from the pathogenic yeast, *Histoplasma capsulatum* [109]. Presumably, this protein localizes in the mitochondria in S. cerevisiae since it naturally has the leader sequence for mitochondrial localization [111]. All eukaryotes, including yeasts, have this alternate respiratory pathway (Figure 5.3) except for S. cerevisiae and Schizosaccharomyces pombe [112,113]. The overexpression of an alternate pathway for NADH oxidation reduced ethanol generation in S. cerevisiae [109], providing even more evidence for excess mitochondrial NADH to cause aerobic ethanol overflow (Figure 5.6). Upon expressing this gene in S. cerevisiae the TCA cycle activity increased, as demonstrated by transcription profiling, proteome studies and enzymatic analysis [109,114]. These two observations: reduction of ethanol and upregulation of the functioning of the TCA cycle upon the introduction of an additional pathway for the oxidation of mitochondrial NADH, indicate that ethanol overflow in S. cerevisiae could be reduced by increasing the respiratory capacity which alleviates NADH-mediated shut-down of the TCA cycle. Increased capacity of the TCA cycle could accommodate more of the glycolytic carbon, which normally would have been shunted to ethanol formation (Figure 5.6).

Figure 0-6

Current model of overflow metabolism in *S. cerevisiae*. There are two overflow metabolites, glycerol and ethanol. Glycerol is produced as a result of cytosolic NADH imbalance, while ethanol is produced indirectly as a result of an imbalance in mitochondrial redox. Alleviation of cytosolic NADH by overexpressing NADH oxidase reduced glycerol generation, while alleviation of mitochondrial NADH reduced ethanol generation [109]. Although a clear genetic

trigger is not yet identified, the proposed mechanism of overflow metabolism explains the observed phenomenon [109].



Perspective

The importance of redox and energetic constraints in governing the product formation is gaining prominence, as reflected by the increasing focus on cofactor engineering. The highly regulated mechanisms of redox homeostasis and consequent energy generation are very well conserved in both *E. coli* and *S. cerevisiae*, barring some subtle differences. Most of these differences can be

attributed to metabolic compartmentalization in S. cerevisiae. For example, acetate is the overflow metabolite in *E. coli*, which is formed by the oxidation of pyruvate (equation 3), whereas ethanol, the overflow metabolite in S. cerevisiae is formed by the reduction of pyruvate (equation 2). Both the mechanisms are the consequence of redox homeostasis. Since flux from acetyl CoA to acetate does not generate any NADH, while the flux from acetyl CoA through the TCA cycle generates NADH, carbon flow diversion to acetate is a means to prevent further NADH accumulation in E. coli. Moreover, the generation of acetate also results in the equimolar ATP generation. On the other hand, S. cerevisiae does not produce acetate as overflow metabolite due to the presence of an extremely efficient pyruvate decarboxylase enzyme [115] which readily converts pyruvate to acetaldehyde. The high affinity of pyruvate decarboxylase towards pyruvate could also be responsible for the conversion of pyruvate to ethanol to consume NADH that is generated in the synthesis of pyruvate. Another step in which NADH could be dissipated in S. cerevisiae is in the conversion of DHAP to glycerol, via glycerol-3-phosphate. This pathway not only involves NADH consumption, but is also associated with ATP generation. Therefore, S. cerevisiae favors glycerol formation to oxidize cytosolic NADH.

Although *E. coli* and *S. cerevisiae* have evolved extremely complex mechanisms to maintain optimal redox coupled with energy synthesis, it is not clear why the regulation of glucose uptake rate has not evolved to correspond with the rate of glucose oxidation capacity. In other words, the rate of glucose uptake could be better concerted with the rate of respiration in order to avoid overflow metabolism. Recently, a new regulatory mechanism of repressing the *ptsG* gene in *E. coli* was discovered [116] that could possibly connect overflow metabolism and glucose uptake. No such regulatory mechanism was reported in *S. cerevisiae*. On the other hand, overflow

metabolism could be seen as an evolutionary advantage in facultative anaerobes to dissipate excess NADH and also as a means to rapidly synthesize ATP. Redox homeostasis, oxidative phosphorylation and respiration are closely linked to metabolism and it is not possible to explicitly distinguish between the cause and effect relationships between them. The critical role they play in industrial and medical biotechnology warrants further research to elucidate the interplay between these three aspects of physiology.

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CHAPTER 6

CONCLUSION

It ain't over.

- Paul Carrack

Conclusion

The fundamental aim of this dissertation was to understand the regulation of overflow metabolism in prokaryotes and eukaryotes using Escherichia coli and Saccharomyces cerevisiae as model organisms, respectively. Overflow metabolism was first described by Crabtree in tumor cells, which produce lactate under aerobic conditions during excess glucose consumption (1) and the phenomenon of aerobic fermentation is known as "Crabtree effect". Subsequently, ethanol was observed in S. cerevisiae fermentations (2) and acetate in E. coli (3) during rapid glucose consumption rates. Although the overflow metabolite differs (lactate in mammalian cells, ethanol in S. cerevisiae and acetate in E. coli), it is believed that the underlying cause is the demand for rapid energy generation. Faster glucose consumption leads to rapid production of ATP in glycolysis which can be used for cellular needs. However, glycolysis also produces NADH which is oxidized in the respiratory chain. The respiratory chain reaches its performance limit, resulting in the accumulation of NADH. Excess NADH is oxidized by dehydrogenases which catalyze metabolic reactions (lactate dehydrogenase, alcohol dehydrogenase or pyruvate dehydrogenase), resulting in the appearance of the corresponding products. The formation of acetate is accompanied with the production of equimolar amount of ATP, providing additional incentive for E. coli to produce acetate under energy-demanding conditions. The formation of glycerol by S. cerevisiae is analogous to acetate formation in E. coli since equimolar ATP is produced in glycerol synthesis. As mentioned in Chapters 4 and 5, aerobic glycerol synthesis in S. cerevisiae is due to excess cytosolic NADH generation. Unlike in S. cerevisiae, ubquinol is the electron donor in the glycerol generation pathway. Therefore, E. coli does not produce glycerol in response to excess NADH. The inability of *E. coli* to produce ethanol using acetaldehyde as an intermediate, a pathway that is extremely efficient in S. cerevisiae, leaves the formation of acetate via acetyl CoA as an energetically efficient means to decrease NADH.

In my doctoral research, experimental data from different levels of metabolism are integrated with physiology and metabolism to deduce the cellular response to genetic and environmental perturbations in *E. coli* and *S. cerevisiae*. This methodology of data integration is now called as systems biology. Despite the new terminology, the concept of integrating data from different cellular components (genes, proteins, metabolites, enzymes, etc) is not new (4). The availability of the genome sequences coupled with the development of high throughput experimental methods for measuring the cellular components has revolutionized systems biology. My thesis represents a unique combination of classical analysis of microbial physiology using bioreactors and controlled growth conditions with the high throughput transcription profiling. This approach enables the identification of the transcriptional changes provide the basis for hypothesis driven metabolic engineering strategies. Chapter 3 illustrates the application of knowledge gleaned from data integration for improving recombinant protein yield in *E. coli*.

With the explosion in biological data, it is only now that mathematical models are being developed that can deal with the data. Mathematical modeling of metabolism based on transcription profiling and networks will provide additional information to cater the metabolic engineer. I hope that my approach of engineering redox fluxes in prokaryotes and eukaryotes will prove to be a valuable tool for the metabolic engineer.

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My pen is at the bottom of a page, Which being finish'd, here the story ends; 'Tis to be wish'd it had been sooner done, But stories somehow lengthen when begun.

- Byron