Chapter 17
Engineering *E. coli* Central Metabolism for Enhanced Primary Metabolite Production

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Contents

Abstract In engineering of *Escherichia coli* for the production of chemicals derived from the central metabolic pathway and in using *E. coli* as a biocatalyst for reactions involving externally supplied specific substrates, there is a need to consider the redox balance and cofactor availability for optimization of the process. Several examples of taking into account the systems biology complexity of redox processes through consideration of gene expression effects, protein level and activity effects, and the role of small molecule effectors of enzyme activity, as well as the role of activation and deactivation of sensitive active site structures are described in the chapter. The manipulation of the availability of reduced cofactors through genetic means and the application of such altered strains for metabolic engineering purposes for the improved production of specific reduced molecules for biofuels, chiral pharmaceutical intermediates, unconjugated colored compounds, and other valuable chemicals is presented.

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17.1 The Central Pathway of *E. coli* Metabolism, a Systems View of the Network and Cofactor Considerations

17.1.1 Aerobic Considerations

Under aerobic conditions the level of reduced cofactors formed in glycolysis and in the TCA cycle can be largely converted to energy for the cell via the electron transport chain and the associated oxidative phosphorylation events. The removal of excess reductant under partial aerobic conditions by an NADH oxidase, particularly an enzyme that forms water, has been demonstrated and can aid flow to more oxidized products in lactic acid bacteria (Lopez de Felipe et al. 1998, Neves et al. 2002). The expression of an NADH oxidase from *Streptococcus pneumoniae* was studied. Results showed that expression of NADH oxidase altered the NADH/NAD+ ratio. In an *arcA* host acetate formation was reduced and the biomass yield increased (Vemuri et al. 2006) suggesting that if the NADH level can be kept low, then the TCA cycle can function efficiently even at a high glucose concentration to process the carbon feedstock without build up of intermediates that generate acetate.

In aerobic processes however, if a redox process for the formation of the desired product is required, the cofactor can be recycled and reduced through the metabolism of a suitable precursor. One also has to consider that the possible utilization of the reduced cofactor through the electron transfer system can compete and limit the availability of the reductant for the desired reaction. In this biocatalyst mode the cells are usually held in a non-growing state, and the aerobically generated reductant can be used more fully in a desired microbial conversion reaction.

The contribution of microaerobic conditions to aid cell energetics and growth properties while allowing more efficient use of carbon for products has also been observed. Early enzyme analysis pointed to factors in the transition (Doelle and Hollywood 1978, Thomas et al. 1972) that were important. It appears the ability to respire oxygen under microaerobic conditions aids *E. coli* in intestinal growth and colonization (Jones et al. 2007). In metabolic engineering practice, a similar strategy is used in the formation of partially oxidized products or where the redox balance would not be appropriate for complete anaerobic metabolism. A number of studies have focused on the contribution that the presence of various oxygen binding proteins such as *Vitreoscilla* hemoglobin can make to enhanced respiration under microaerobic conditions and the effects on cell physiology, productivity, and metabolic pattern (Andersson et al. 2000, Frey et al. 2000, Kallio et al. 1996). Studies of the relative expression of genes (Liu and De Wulf 2004, Overton et al. 2006, Salmon et al. 2003) and metabolite patterns under conditions of limited oxygen have been made with wild type and various metabolic and regulatory mutant strains under defined oxygen conditions (Alexeeva et al. 2000, 2002, 2003, Becker et al. 1997, Partridge et al. 2007, Shalel-Levanon et al. 2005a,b,c, Zhu et al. 2006, 2007a). Such measurements have allowed models of the shift between aerobic and anaerobic conditions to be formulated and their general features to be evaluated (Govantes et al. 2000, Peercy et al. 2006, Schramm et al. 2007). The metabolite pattern of

products derived from pyruvate arising in various mutant strains under conditions of low oxygen is complicated by many factors influencing the *in vivo* activities of the various enzymes around this node. For example the activities of Pdh and Pfl are affected by gene expression levels, the NADH level and the relative amounts of activation, deactivation of Pfl as well as the YfiD interaction with Pfl. The levels of other enzymes acting around the pyruvate node and the TCA cycle and cytochrome oxidase enzymes also influence the level of small molecules that can affect *in vivo* activity and metabolic flux through the competing routes. Some discussion of these influences is given in (Peercy et al. 2006, Shalel-Levanon et al. 2005b, Zhu et al. 2007a) (Fig. 17.1).

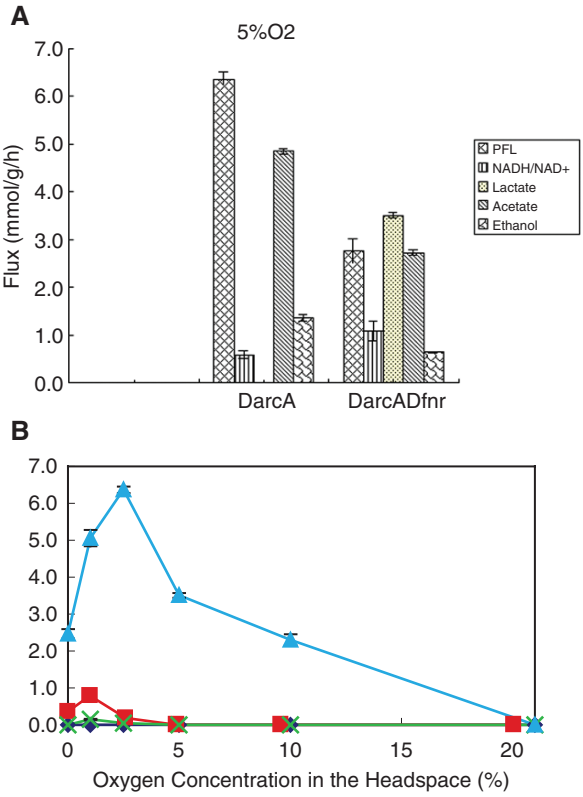

Fig. 17.1 (**a**) Comparison of metabolites and fluxes of cultures of MG1655 DarcA (*arcA* disruption) and MG1655 DarcA, Dfnr strains grown in chemostat under 5% oxygen in the headspace. The difference in lactate flux is most apparent. Other fluxes are shown as indicated. The NADH/NAD+ ratio is also shown. In the parent, MG1655 the other metabolites were not observed see Fig. 17.1b. (**b**) Metabolite fluxes as a function of the oxygen concentration in the headspace at steady state. PFL, lactate, ethanol, and succinate. The fluxes in the individual strains are indicated: (purple, dark gray diamond) MG1655, (red,dark gray squares) MG1655 [DarcA], (green, light gray x) MG1655 [Dfnr], (blue, light gray triangles) MG1655 [DarcA, Dfnr]. The error bars indicate the standard deviation of three samples taken after 7, 7.5, and 8 residence times

17.1.2 Anaerobic Considerations

Under anaerobic growth the reductant formed in glycolysis must be recycled by reactions using available substrates. This process generates the reduced metabolites derived from pyruvate in many bacterial species and the reduced products of the mixed acid fermentation in *E. coli*. By limiting the alternative pathways for cofactor recycling, the metabolic course of the flux into the downstream parts of the central pathway is affected. The dissipation of the reducing equivalents can also be handled through the formation of hydrogen either directly or through the release of a compound such as formate which can easily be converted to hydrogen and carbon dioxide. Bacteria have elaborate sensing mechanisms for oxygen and regulate the specific cytochrome oxidases as well as many other genes through transcriptional regulators such as ArcA and Fnr. The area of aerobic/anaerobic gene regulation mechanisms will not be covered here as it is reviewed elsewhere in this volume and in other reports (Gunsalus and Park 1994, Sawers 1999). The various electron carriers; flavins, nucleotide cofactors, quinones and ferredoxins, act with specific enzymes and while there is interconversion among the reduced compounds the redox potential and relative quantity of each within the cell suggests a distinct role for the individual carriers in the cell. The efficiency of rapid equilibrium among the pools of reduced electron carriers is dependent on a number of factors including the relative location in the cell, association of key molecules with other cell components, and specific binding constants and kinetic parameters of competing reactions. These factors can be adjusted by engineering but the physiological response of the cell is often complicated.

17.2 Strategies for Engineering Metabolic Outputs from Specific Branches

17.2.1 Multiple Deletions in Alternative Pathways

17.2.1.1 Pyruvate and Acetate

Pyruvate is formed under aerobic conditions when it is desired to produce it in high quantity (Causey et al. 2004, Sakai et al. 2007, Tomar et al. 2003, Zelic et al. 2006, 2004a,b). Some similar features have been implemented in the high production of acetate by *E. coli* (Causey et al. 2003). The general strategy for high production of these compounds involves high glycolytic fluxes and the removal of competing pathways, either for the carbon or for the reductant in order to minimize the potential formation of further metabolism of the compound (e.g. pyruvate conversion to lactate). In the case of acetate production, the elimination of reactions involving a key precursor (e.g. pyruvate conversion to other products) can affect the yield and culture performance. Since these compounds are dealt with elsewhere in this volume the specifics of metabolic engineering of *E. coli* for production of these products will not be discussed here.

One area of interest related to industrial production is the reduction of acetate formation that can inhibit growth and limit productivity in a variety of processes including recombinant protein production. Several strategies have been investigated to avoid acetate formation. In cultures, limited glucose addition can avoid some of the problems but requires careful control of the culture. These engineering strategies have become widely practiced as computer controls and sensors have become more sophisticated but are still a concern for optimization and reproducibility in large scale processes. The reduction of glucose uptake and the avoidance of build-up of the glycolytic intermediate, pyruvate, can be accomplished via genetic changes affecting the glucose transport system (Backlund et al. 2008, Chen et al. 1997, De Anda et al. 2006, Hernandez-Montalvo et al. 2003, Lara et al. 2008, Picon et al. 2005, Wong et al. 2008, Yi et al. 2003) or the presence of modified sugars (Aristidou et al. 1999, Chou et al. 1994). A large number of studies on the effects of *ptsG* mutations on production of acetate and other compounds, recombinant proteins, and growth have shown the importance of coordination of glucose uptake with downstream metabolism to avoid excessive acetate production and performance limitations.

The inactivation of genes that encode the major acetate formation pathway enzymes (acetate kinase, *ack* and phosphotransacetylase, *pta*; and pyruvate oxidase, *poxB*) can relieve acetate formation (De Mey et al. 2007) although such mutations may reduce the growth rate under some conditions or in certain genetic backgrounds (Abdel-Hamid et al. 2001, Flores et al. 2004, Vemuri et al. 2005). The effects of fluctuations in oxygen on the formation of acetate and recombinant proteins has been examined with the observation that the genes of fermentative metabolism can be removed with accompanying improved performance (Lara et al. 2006). The differences in *E. coli* strains have been studied and the flux through the glyoxyate pathway, acetate uptake and synthesis, and gluconeogenesis were different among some widely used laboratory strains and accounted for the differences in acetate formation in cultures of *E. coli* B and JM109 (Phue et al. 2005) and the extent of flux through anaplerotic pathways influences acetate excretion (Farmer and Liao 1997). Acetate formation can also be addressed through diversion of the precursor, pyruvate, to a non-toxic compound such as acetoin by incorporation of the gene encoding an acetolactate synthase from another organism (Yang et al. 1999).

17.2.1.2 Lactate

While lactate is readily formed by lactic acid bacteria and other microbes, it is formed naturally in differing amounts by various *E. coli* strains. Lactate formation in *E. coli* has been engineered, with either stereoisomer being formed depending on the particular characteristics of the lactate dehydrogenase employed (Chang et al. 1999, Dien et al. 2001, Fong et al. 2005, Hua et al. 2006, Zhou et al. 2003a,b, Zhou et al. 2005, Zhu et al. 2007b). In this case the fermentation is anaerobic and the other pathways that could use the reduced cofactor formed in glycolysis are removed (e.g. pyruvate conversion to acetyl-CoA and subsequently on to ethanol). Efficient natural production of this compound by other organisms is available and several engineered *E. coli* strains also perform well.

17.2.1.3 Succinate

In the case of succinate production, the conversion of glycolytic intermediates to oxaloacetate is a key step and in order to obtain high conversion enzymes capable of forming OAA or malate (Hong and Lee 2001, Kim et al. 2004, Lin et al. 2004, 2005e, Sanchez et al. 2005b, Stols et al. 1997, Vemuri et al. 2002) are usually overexpressed either through recombinant techniques or by enhancement of the natural system. There is a two fold problem in attaining the highest possible molar yield from glucose; one is the limitation of reductant (Hong and Lee 2002), if the 2 molecules of NADH formed in glycolysis are used to reduce the oxaloacetate, only one molecule of succinate can be formed. There is an alternative way to form succinate that does not consume NADH, i.e. through the glyoxylate route of the TCA cycle. This route also is limited to production of one molecule of succinate from one glucose due to loss of carbon in this normally aerobic pathway (Lin et al. 2005a,c,d). The correct partitioning of oxaloacetate between the reductive and oxidative routes can increase the overall yield while maintaining the NADH balance (Cox et al. 2006, Sanchez et al. 2006).

A variety of mutations to route the products of glycolysis to succinate have been investigated with the effects of redox systems (Yun et al. 2005) and the sugar uptake system (Chatterjee et al. 2001, Wang et al. 2006) showing a significant effect in some backgrounds due to the effects on pyruvate formation. Performance on various hexose and pentose sugars have been studied with glucose generally offering the highest yield compared to fructose or xylose (Andersson et al. 2007, Lin et al. 2005b). Computational methods have also been employed to identify high yielding strains (Lee et al. 2005) or model the immediate metabolic network (Cox et al. 2006). Strains made with an idea of optimal succinate production have included those with a number of defined mutations (Sanchez et al. 2006) and evolved strains derived from a defined parent (Jantama et al. 2008). In the studies various experimental conditions have been examined with the key factors of yield from feedstock, rate of production, productivity per cell mass, and final titer being components of the calculation of the potential of the process.

17.2.2 Alteration of Cofactor Availability (NADH)

Efforts to modify the NADH availability for cell metabolism have been undertaken for many years and have been based on observations of differing metabolic products formed using similar sugars with different oxidation levels such as glucuronic acid, glucose, and sorbitol. In these cultures the pattern of products formed, acetate, ethanol, formate, lactate, and succinate changes with the more oxidized products dominating in the culture from the oxidized substrates and the more reduced products being enhanced upon culture growth on sorbitol, a more reduced substrate. More oxidized products can be formed by depleting the NADH by an NADH oxidase as mentioned above. Here we will consider the changes in metabolites when an effort is made to augment the normal amount of NADH produced by the wild type *E. coli* strain.

Manipulation of the conversion reaction of pyruvate to acetyl-CoA and the subsequent release of formate, if formed, can alter the amount of reductant available to the cell. The production of NADH will favor the formation of more reduced products. There are three general enzymes that can catalyze this reaction each giving its own reduced product; pyruvate dehydrogenase that forms NADH and acetyl-CoA (Cassey et al. 1998, Guest et al. 1981, 1989, Guest and Stephens 1980, Haydon et al. 1993), pyruvate ferredoxin oxidoreductase that forms reduced ferredoxin or flavodoxin and acetyl-CoA (Blaschkowski et al. 1982, Reed et al. 2003, Serres et al. 2001), and pyruvate formate lyase that forms formate and acetyl-CoA (Birkmann et al. 1987, Knappe and Blaschkowski 1975, Knappe et al. 1984, Knappe and Sawers 1990, Pecher et al. 1982, Sauter and Sawers 1990, Sawers and Bock 1988, Varenne et al. 1975) with a number of articles describing the free-radiacal enzyme and its activation under anaerobic conditions and inactivation under aerobic conditions and the participation of proteins such as AdhE, YfiD and PflA in defining the activity of the protein (Becker et al. 1997, 1999, Chase and Rabinowitz 1968, Hoover and Ludwig 1997, Knappe and Wagner 1995, Kulzer et al. 1998, Nnyepi et al. 2007, Reddy et al. 1998, Sawers et al. 1998, Sawers and Watson 1998, Wagner et al. 2001, Zhang et al. 2001, Zhu et al. 2007a). If the reaction gives rise to NADH directly the reduced nucleotide cofactor can be used for production of a desired reduced product. The pyruvate dehydrogenase is generally the active enzyme under aerobic conditions and it is replaced by the pyruvate formate lyase under limiting oxygen conditions. The pyruvate dehydrogenase can still operate under anaerobic conditions, however high NADH is often inhibitory to the reaction (Snoep et al. 1993, Zhu et al. 2007a). The role of PdhR in regulating the Pdh system and effects of mutations of *pdhR* on expression and metabolism have been studied (Haydon et al. 1993, Kim et al. 2007, Ogasawara et al. 2007, Quail and Guest 1995, Zhou et al. 2008). As an added feature, the production of formate, while the final step under neutral pH conditions by Pfl, formate is further hydrolyzed to hydrogen and carbon dioxide under acidic conditions by the formate hydrogen lyase system (Bagramyan and Trchounian 2003, Birkmann et al. 1987). This reaction thereby removes the acidic metabolite formate but does not generate any useful reductant or energy for the cell but could reduce some acid stress due to formate accumulation. The effect of formate hydrogen lyase and other hydrogenases has been studied with regard to hydrogen production (Maeda et al. 2007a, Redwood et al. 2008, Yoshida et al. 2005, 2007). In some cases an uptake hydrogenase can recapture a portion of the hydrogen released and it can thereby affect the pattern of metabolites (Francis et al. 1990, Laurinavichene and Tsygankov 2001, Maeda et al. 2007b, Redwood et al. 2008).

The reducing equivalents available in formate can be recaptured to NADH rather than be released to hydrogen by incorporation of a NADH-dependent formate dehydrogenase (Berrios-Rivera et al. 2002a,b, Galkin et al. 1997, Sanchez et al. 2005a, Slusarczyk et al. 2000). Such NADH coupled enzymes are known in a number of organisms and those of Candida have been used *in vitro* and *in vivo* for regeneration of the NADH pool. Optimal enzymes from *Candida boidinii* and *Mycobacterium vaccae* that are more stable have been generated by mutation (Slusarczyk et al. 2000, Tishkov and Popov 2006, Yamamoto et al. 2005) and NADH-dependent formate

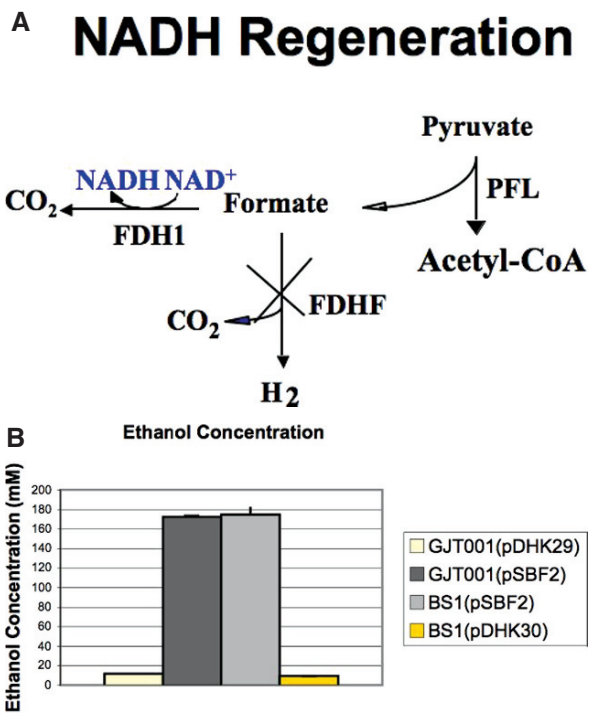

Fig. 17.2 (**a**) NADH coupled formate dehydrogenase pathway. The native NAD independent formate hydrogen lyase pathway uses (FDHF: formate dehydrogenase, NAD independent) to convert formate to hydrogen and carbon dioxide. The newly added NAD+ dependent pathway (in blue, light gray) uses (FDH1: NAD+ dependent formate dehydrogenase, FDH1 encoded by *fdh1* from *Candida boidinii*) to convert formate to carbon dioxide and the reduced cofactor NADH. (**b**) Effects on ethanol formation of expression of a NADH-dependent formate dehydrogenase in *E. coli*. The *E. coli* strain GJT001 is a W3110 derivative parental strain and BS1 has an inactivated *fdhF* gene. The plasmid pDHK29 is the vector and pSBF2 contains the *fdh1* gene from *Candida boidinii*. Growth was in L-broth plus 20 g/L glucose

dehydrogenases from other organisms have been isolated (Nanba et al. 2003a,b). Such enzymes are used to recycle NADH for use in formation of valuable compounds such as the pharmaceutical precursor, ethyl (S)-4-chloro-3-hydroxybutanoate (Yamamoto et al. 2005). The formation of chiral pharmaceutical intermediates using NADH regeneration has been reviewed (Patel 2000) (Fig. 17.2).

17.2.2.1 Ethanol

The capture of all available reducing power from glycolysis and present in pyruvate is needed for optimal formation of 2 molecules of ethanol from glucose. In *E. coli* such high formation of ethanol has been achieved through the addition of the *pdc* and *adh* genes from *Zymomonas mobilis* (Ingram et al. 1987, 1999, Jarboe et al. 2007). The recapture of the reductant in formate via a NADH-dependent formate

dehydrogenase can also give essentially complete conversion of glucose to ethanol (Berrios-Rivera et al. 2002b, 2004) and chemostat cultures have shown the effect on metabolites using different carbon sources (Sanchez et al. 2005a).

17.2.2.2 *E. coli* Cells as Single Step Biocatalysts

The use of regenerated NADH to carry out a reduction by a whole cell biocatalyst has some advantage over using a purified enzyme in that the cell takes care of the recycling step and the cofactor is confined within the cell. Several papers have used such recycling systems in roles as cellular biocatalysts for amino acid (Galkin et al. 1997) and mannitol production (Kaup et al. 2003, 2004, 2005).

17.2.3 Alteration of Cofactor Availability (NADPH)

The pentose phosphate pathway, *zwf* and isocitrate dehydrogenase, *icd* are generally considered to be the major sources of reductant NADPH which is used in many biosynthetic reactions. The preference for NADPH can limit the production of the desired product since the NADPH pool is considerably smaller than the pool of NADH. Efforts to enhance the equilibration between the two reduced nucleotide cofactors has been investigated. There are two transhydrogenases in *E. coli*, *udhA* (*sthA*) and *pntAB*. The proton-translocating transhydrogenase PntAB was identified as the major source of NADPH under aerobic growth with the pentose phosphate pathway contributing almost as much and isocitriate dehydrogenase making up most of the remainder. While the energy-independent transhydrogenase UdhA (SthA), seemed to be essential under metabolic conditions with excess NADPH formation suggesting it played more of a role in dissipating NADPH to NADH (Sauer et al. 2004). Alterations of the transhydrogenase do indeed increase the level of NADPH-dependent products that are formed (Weckbecker and Hummel 2004). Another strategy to produce more NADPH for a conversion is to use a biocatalyst with a special system and substrate for producing NADPH based on the oxidation of the specific exogenous added substrate by a NADPH-dependent redox enzyme and the use of the NADPH for synthesis of the desired reduced product (e.g. a chiral alcohol). Another approach is to guide more metabolism through the pentose phosphate pathway where NADPH is formed in an early step. Several papers have analyzed the effects of mutations affecting glycolytic enzymes or overexpression of glucose-6-phosphate-1-dehydrogenase, *zwf*, in the context of NADPH usage. A discussion in consideration of the effects on PHB production is given below.

A more recent strategy is to incorporate a NADPH-utilizing step to replace a natural NADH-dependent step in glycolysis. This approach of using an NADPH-utilizing enzyme from another organism can provide additional NADPH for use by an added pathway that consumes high amounts of the cofactor (a NADPH sink). Several pathways utilize NADPH in high amount such as those for the biodegradable polymer, polyhydroxybutyrate and many unsaturated colored compounds and terpeniod compounds derived from the isoprenyl pyrophosphate pathway. Naturally

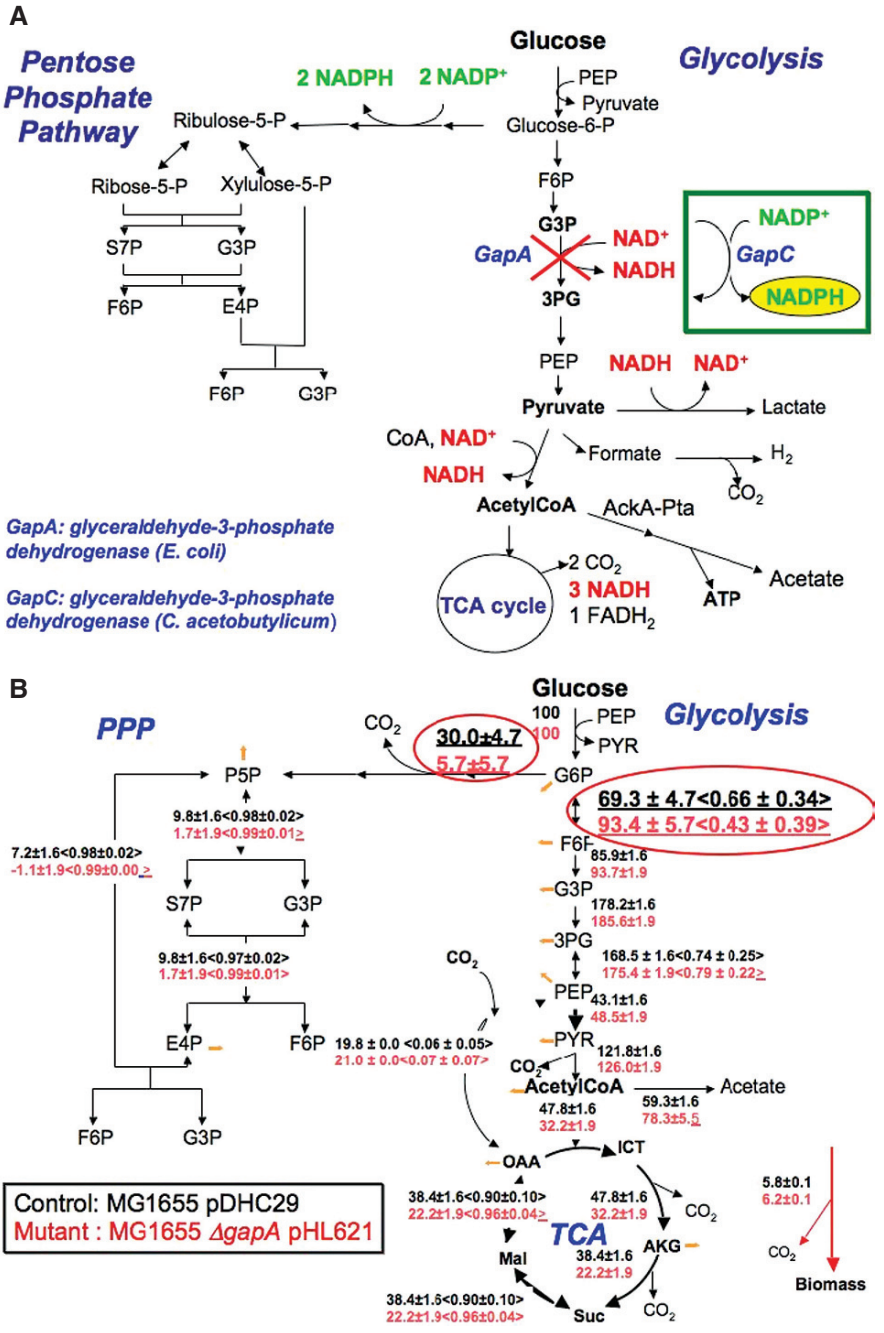

Fig. 17.3 (continued)

existing pathways in *E. coli* or specialized pathways can be introduced to assess the effects of manipulation of NADPH on the production of these compounds. Frequently NADPH is used as a recycling compound in combination with oxidative metabolism, such as with P450 type enzymes and monooxygenases, and studies can examine the efficiency of NADPH recycling systems on processes catalyzed by such enzymes (Fig. 17.3).

17.2.3.1 PHB

The pathway to PHB and other polyhydroxylalkanoates uses NADPH in the reduction step of the individual monomers (Saito et al. 1977) and since a large amount of this product can be formed in engineered *E. coli*, it can serve as a useful test system for accessing the effects of attempts to alter NADPH availability. There have been many studies of the production of PHB type molecules in *E. coli* (Fidler and Dennis 1992, Lee et al. 1994, Peoples and Sinskey 1989, Schubert et al. 1988, Slater et al. 1988, 1992, Timm and Steinbuchel 1992) and recent reviews have appeared (Dias et al. 2006, Keenan et al. 2006, Nomura and Taguchi 2007, Rehm 2007, Steinbuchel 2005, Steinbuchel and Hein 2001). The influences of various approaches are discussed below.

The inactivation of the *talA* gene increased PHB content and effect was thought to arise from effects on supplies of the intermediates NADPH and acetyl-CoA (Song et al. 2006) and a similar effect was noted upon overexpression of the *tktA* gene (Jung et al. 2004). Directly overexpressing *zwf* encoding glucose-6-phosphate dehydrogenase increased PHB accumulation (Lim et al. 2002). These alterations of the pentose pathway would promote increases in the major precursors. Efforts have been made to engineer additional NADPH availability by processing more of the glucose through glucose 6-phosphate dehydrogenase by using a mutation causing *pgi* gene inactivation. NADPH overproduction through the pentose phosphate pathway in the *pgi* mutant strain causes some reducing power imbalance that ultimately can affect the cell growth (Kabir and Shimizu 2003a,b). Experiments analyzing the concentrations of intermediates and coenzyme ratios acetyl-CoA/CoA, total CoA, and NADPH/NADP ratios showed that the PHB flux was highly sensitive to the acetyl-CoA/CoA ratio (response coefficient 0.8), total acetyl-CoA + CoA concentration (response coefficient 0.7), and pH (response coefficient −1.25) (van Wegen et al. 2001). It was less sensitive (response coefficient 0.25) to the NADPH/NADP ratio. The total NADP(H) concentration (NADPH + NADP) had a negligible effect.

Fig. 17.3 (**a**) The pathway diagram shows the formation of NADPH in the pentose phosphate pathway and the modification of the glycolytic pathway by replacement of the normal *gapA* by a *gapC* gene from *C. acetobutylicum*. The GapC can form NADPH and lead to increased availability of NADPH. (**b**) Metabolic flux distribution in control and modified *E. coli* strains. The data in the figure indicate the net flux values in *E. coli* strains calculated from steady state cultures and C-13 labeling experiments. In the top row is shown the values for *E. coli* MG1655 (pDHC29, the vector) and the corresponding values from cultures of the *E. coli gapA* mutant strain harboring the plasmid pHL621 containing *gapC* from *Clostridium acetobutylicum* are shown in the second row. The values in brackets represent the exchange coefficients of the fluxes (Martinez et al. 2008)

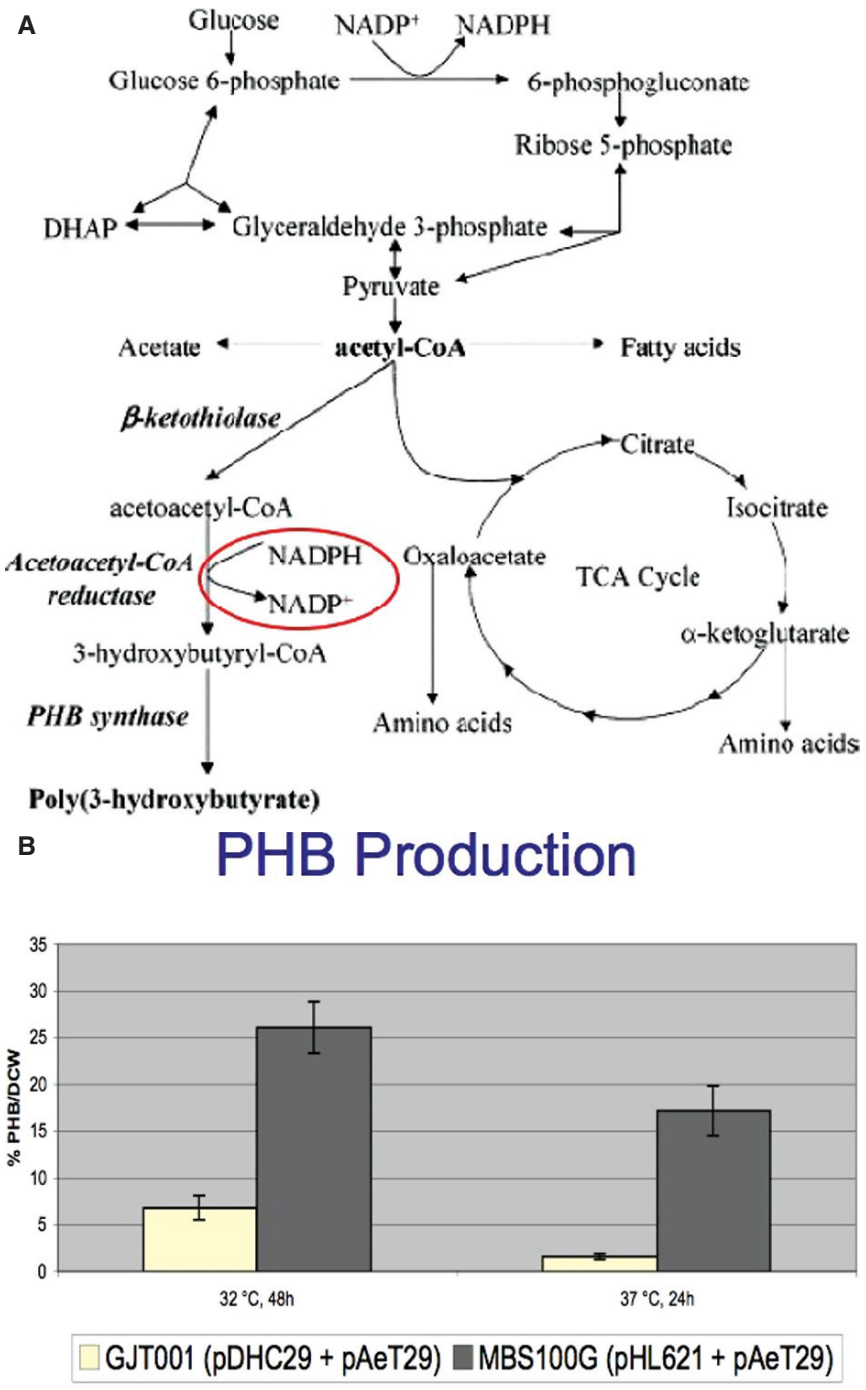

Fig. 17.4 (continued)

The effect of *pta* inactivation on PHB synthesis was studied in cultures grown on several media with the observation that a decrease in Pta activity probably causes some increase in acetyl-CoA as substrate for the PHB synthesis pathway, resulting in increased PHB accumulation (Miyake et al. 2000). The effects of *ack-pta* and *pgi* mutations on PHB synthesis was studied (Shi et al. 1999) and the improved performance of the strain with the *pgi* mutation was observed, however the effect of the alteration of acetyl-CoA suggested it was not so important in that situation.

A strain with altered NADPH availability was tested for PHB production. In this strain the normal NADH-utilizing *E. coli* GAPDH was replaced with a NADPH-utilizing enzyme from *Clostridium acetobutylicum* (Martinez et al. 2008). PHB experiments were performed at 32 °C and 37 °C until glucose was exhausted. Cells grew slower at 32 °C but higher amounts of PHB were produced. After 48h, the modified *E. coli* produced 26% of PHB/DCW compared to 6.8% of PHB/DCW of the control, showing an increase of 3.8-fold. The mutant strain of *E. coli* also produced a significantly higher amount of PHB at 37 °C compared to the control (11-fold) but the final concentration was lower than at 32 °C. These results showed that the *gapA* mutation and introduction of the *gapC* gene did increase the PHB production and further indicated the key role of NADPH availability in allowing high PHB production (Fig. 17.4).

17.2.3.2 Lycopene

Lycopene, a highly unsaturated compound of interest for its color and food ingredient properties, consumes a large amount of NADPH during its biosynthesis. Lycopene synthesis has been studied in *E. coli* with overexpression and engineering of genes of the pathway (Alper et al. 2006, Cunningham et al. 1994, Kim et al. 2008, Kim and Keasling 2001, Linden et al. 1991, Misawa et al. 1990, Misawa and Shimada 1997, Sandmann et al. 1990, Vadali et al. 2005, Wang et al. 2000, Yoon et al. 2006, 2007a,b) and chemical variations of the basic carotenoid compounds have also been formed in *E. coli* (Gallagher et al. 2003, Kajiwara et al. 1997, Lee et al. 2003, Schmidt-Dannert et al. 2000). A variety of approaches have been used to improve production. These include the overexpression of chromosomal genes of *E. coli* by the insertion of strong promoters to direct high level of expression of selected genes (Alper et al. 2005a) or addition of plasmids bearing these genes (Kang et al. 2005). The idea of balance among the levels of various gene products in generating high flux through the pathway while avoiding build-up of any toxic intermediates is a factor in this sort of pathway (Farmer and Liao

Fig. 17.4 (**a**) The pathway for production of PHB. The diagram shows the requirement for NADPH in reduction of the intermediate for polymerization of the PHB precursor. (**b**) Aerobic PHB production by control and *gapA* mutant *E. coli* strain overexpressing the *gapC* gene from *C. acetobutylicum*, both control and modified strains harbor the *phb* operon from *Alcaligenes eutrophus* for PHB synthesis. Control strain: GJT001 (pDHC29 + pAeT29); *gapC* containing mutant strain: MBS100G (pHL621 + pAeT29)

2000, Farmer and Liao 2001, Matthews and Wurtzel 2000, Smolke et al. 2001). As an example Farmer and Liao (Farmer and Liao 2000) manipulated precursor availability to increase lycopene production, they showed the G3P pool could be a limiting factor in their system. The effects of mutations on the synthesis of lycopene have been investigated by computational and experimental approaches (Alper et al. 2005b, Alper and Stephanopoulos 2008, Hemmi et al. 1998, Jin and Stephanopoulos 2007). In recent studies, a large number of individual mutations were screened and several genes were overexpressed in the host. Then combinations of mutations with improved performance were genetically combined to generate a strain with substantially greater production. This type of survey of the metabolic landscape identified the best-engineered strain (T5(P)-*dxs*, T5(P)-*idi*, *rrnB*(P)-*yjiD*-*ycgW*, delta *gdh* delta *aceE* delta *fdhF*, pACLYC), Further study

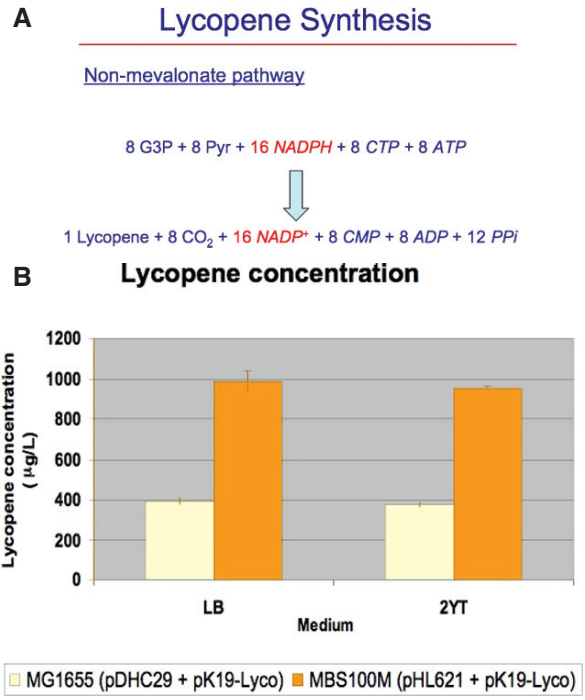

Fig. 17.5 (**a**) Lycopene synthesis by the non-mevalonate pathway requires a high amount of NADPH. (**b**) Effect of increased NADPH availability on lycopene production. The final lycopene concentration of control and modified *E. coli* strains after aerobic culture is shown. The cultures were grown in LB or 2YT medium supplemented with 20 g/L of glucose for 24h at 30 °C and 250 rpm. The data shown are the average of three replicate experiments where the error bars represent the standard deviation. Control strain: MG1655 (pDHC29, + pK19-Lyco); modified strain: MSM (pHL621 + pK19-Lyco). pDHC29 is the control vector and pHL621 carries the *gapC* gene coupling NADPH formation to the glycolytic pathway. pK19-Lyco carries the lycopene biosynthetic pathway genes (Cunningham et al. 1994)

with a large number of mutations demonstrated the complexity of mapping only one genotype to one phenotype. The investigation of combinations identified a particularly interesting mutant, the $\Delta hnr\Delta yliE$ genotype, that exhibited a drastically improved lycopene production (Jin and Stephanopoulos 2007, Alper and Stephanopoulos 2008).

The effects of the above manipulation of NADPH forming pathway, GAPDH alteration, on the levels and productivity of the strains has also been explored. The cell growth of the altered *E. coli* strain was comparable to the parental control and no growth impairment was detected. A significant difference was found in lycopene production between the two strains. The NADPH altered strain produced

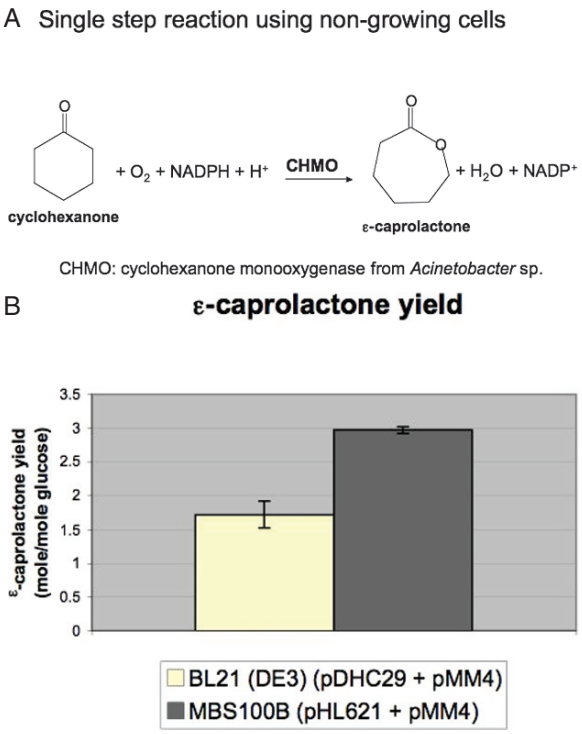

Fig. 17.6 (**a**) Synthesis of ε-caprolactone in recombinant *Escherichia coli* expressing cyclohexanone monooxygenase (CHMO) from *Acinetobacter* sp. (**b**) Effect of increased NADPH availability on conversion of cyclohexanone to the lactone. The final lactone concentration of control and modified *E. coli* strains after aerobic culture is shown. The cultures were grown in LB medium and the expression of CHMO was induced with IPTG. After reaching stationary phase the cells were was re-suspended in 20 ml of non-growing medium containing glucose and 30mM cyclohexanone and incubated for 20 h. Concentrations of cyclohexanone and ε-caprolactone were analyzed. Control strain: BL21 (DE3) contains (pDHC29, + pMM4); the BL21 gapC modified strain: MBS 100B contains (pHL621 + pMM4). pDHC29 is the control vector and pHL621 carries the *gapC* gene coupling NADPH formation to the glycolytic pathway. pMM4 carries the cyclohexanone monooxygenase (CHMO) from *Acinetobacter* sp (Walton and Stewayt 2002)

lycopene equivalent to 2.5-fold that of the control in concentration. The overexpression of the NADPH-utilizing GAPDH from *C. acetobutylicum* together with the knockout of the native NADH-dependent GAPDH improved lycopene synthesis confirming that cofactor availability is a limiting factor for the system (Fig. 17.5).

17.2.3.3 Single Step Biocatalyst

In the area of using engineered *E. coli* as a whole cell biocatalyst for a specific conversion, the emphasis has been on placement of an oxidizing step into the cell and supplying the cell with the substrate. In optimal cases the product of the oxidation step is easily removed from the reaction. The NADPH formed in this step is then used to provide the reductant for the synthesis of the desired product. A useful example of this has been studied using the recycling of mono-oxygenases to form lactones particularly chiral derivatives. In this kind of test system using a strain in which the replacement of a normal glycolytic step using NAD with one capable of using NADP, a positive effect was seen on the production rate and the amount of desired compound formed per mole of glucose consumed. The mutant host strain containing the clostridial GAPDH gene showed a higher ε-caprolactone yield that of the control strain, 2.97 compared to 1.72 mole ε-caprolactone/mole glucose. One mole of NADPH is consumed per mole of ε -caprolactone produced; therefore the mutant strain produced 73% more NADPH than the control strain under the conditions examined (Fig. 17.6).

17.3 Conclusions

In a variety of studies, it has been shown that considerable changes in metabolic pattern can be achieved by manipulation of the availability of the oxidation-reduction cofactors, NADH and NADPH. The alteration in availability of CoA compounds has also indicated that this approach can offer improvements in the synthesis of compounds derived from central pathway CoA containing intermediates. The addition of these cofactor manipulations to the arsenal of metabolic engineering tools should expand the sophistication of cell engineering as well as allow a greater understanding of the role of the various redox carrier systems and activated carriers in cell metabolism and physiology.

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