Chapter 8
Metabolic Reprogramming Under Microaerobic and Anaerobic Conditions in Bacteria

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Abstract Oxygen has a great impact on the metabolism and physiology of microorganisms. It serves as the most efficient terminal electron acceptor to drive the energy conservation process of cellular respiration and is required in many biosynthetic reactions. Bacteria encounter oxygen fluctuation and limitation during their growth in both natural ecological niches and in laboratory vessels. In response to oxygen limitation, facultative bacteria undergo substantial metabolic reprogramming to switch from the aerobic respiration to either anaerobic respiration, fermentation, or photosynthesis. Two key factors determine the metabolic pathways bacteria adopt under oxygen deprived microaerobic and anaerobic conditions: maximal energy conservation and redox homeostasis. In this chapter, we first describe how the fulfillment of these two key factors governs the metabolic reprogramming of facultative bacteria and how the process is tightly controlled by several global regulatory factors: FNR, ArcBA, as well as NarL and NarP. We then utilizes fermentation of glycerol, a large surplus byproduct of biodiesel industry, as an example to illustrate how environment, process, and strain based approaches can be exploited to manipulate and engineer the anaerobic metabolic pathways so that desirable fermentation products can be achieved with optimal yield.

Keywords Aerobic respiration • Anaerobic respiration • Fermentation • Glycerol fermentation • Redox homeostasis

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Abbreviations

Arc	aerobic respiratory control
ATP	adenosine-5′-triphosphate
ETC	electron transport chain
FNR	fumarate and nitrite reduction
NADH	nicotinamide adenine dinucleotide (reduced form)
TCA cycle	tricarboxylic acid cycle

8.1 Introduction

Oxygen (O_2) as an atmospheric component has a great impact on the life style of living organisms on earth. It serves as the most efficient terminal electron acceptor to drive the energy conservation process of cellular respiration and is required in many biosynthetic reactions. Owing to its substantial effect on the growth and metabolism of microorganisms, how these organisms respond to the presence and levels of O_2 has been an important scenario to categorize microbial species. From this regard, microorganisms are classified as obligate aerobes, facultative anaerobes, aerotolerant anaerobes, microaerophiles, and obligate anaerobes. Facultative anaerobes are those that can grow both in the presence (aerobic) and absence (anaerobic) of O_2 and have been an important class of organisms exploited in biotechnology owing to their broad growth conditions and metabolic diversities. In order to grow both in the presence and absence of O_2 in their ecological niches, these microorganisms have evolved complex strategies to optimize their metabolic efficiencies in response to the changes of oxygen levels. In laboratory cultivation of bacteria for research or biotechnological purposes, fluctuation of oxygen levels in growth vessels likewise has a significant impact on the physiological status of bacteria and consequently the types of metabolic products they generate. Understanding this metabolic reprogramming process of bacteria in response to oxygen availabilities thus is essential for the design and optimization of bacterial cultivation systems for research and biotechnological applications. In this chapter, we first summarize the fundamental principles that govern the metabolic reprogramming of facultative anaerobic bacteria during their switch from aerobic to microaerobic or anaerobic conditions, and then discuss various factors and approaches that can be utilized to optimize the metabolic reprogramming processes in biotechnological applications.

8.2 Key Factors That Govern the Metabolic Reprogramming of Bacteria in Response to O_2

Two major requirements govern the metabolic reprogramming for facultative bacteria in response to different O_2 levels: to achieve optimal energy conservation and to maintain the cellular redox homeostasis. Under aerobic growth conditions, facultative

anaerobic bacteria conserve energy mainly by aerobic respiration, during which organic carbon sources are converted to CO_2 and the resulting reducing equivalents (NADH and $FADH_2$) are oxidized by the terminal electron acceptor O_2 through the aerobic electron transport chain (ETC). ATP is simultaneously generated during this process by means of oxidative phosphorylation. Under anaerobic conditions bacteria must adjust their strategies for energy conservation and redox homeostasis owing to the lack of O_2 as the terminal electron acceptor of the ETC. Under this condition the energy generation pathways adopted by bacteria include anaerobic respiration, fermentation, or photosynthesis. For chemoorganotrophic and chemolithotrophic bacteria, ATP can be yielded from anaerobic respiration or fermentation. For facultative phototrophic bacteria, such as purple non sulfur bacteria, usually photosynthesis is adopted upon the reduction of oxygen concentration. Concomitant with the energy production, NADH generated during the breakdown of the reduced carbon sources are recycled to NAD^+ in either of the processes of anaerobic respiration, fermentation, or CO_2 fixation depending on the species utilized and the availability of external electron acceptors (McEwan 1994). Thus, cellular redox balance is achieved. Since chemoorganotrophic bacteria represent the majority of the species utilized in biotechnology, we next use this class of microorganisms as an example to illustrate the metabolic reprogramming of bacteria in response to O_2 availabilities.

8.3 Aerobic Metabolism of Facultative Anaerobic Bacteria

Under aerobic growth conditions with sufficient carbon sources, aerobic respiration is adopted by facultative anaerobes to conserve energy owing to the highest yield of ATP through this process. The process of aerobic respiration can be divided into carbon flow and electron transport through the electron transport chain. Bacteria will first convert carbon sources such as monosaccharides, amino acids, short chain alcohols or acids to pyruvate. This process usually generates a limited number of ATP and reducing equivalents, often in the form of NADH. A typical example of this conversion is the degradation of glucose to yield pyruvate, referred as glycolytic pathways or glycolysis (Fig. 8.1), which is found in all major groups of microorganisms and functions both in the presence or absence of O_2. During aerobic respiration, pyruvate is converted to acetyl-CoA and CO_2 by the pyruvate dehydrogenase complex (PDHC). Acetyl-CoA then enters the tricarboxylic acid (TCA) cycle and be oxidized to CO_2. The various intermediates in the TCA cycle can be supplied to anabolic pathways for biosynthesis (Fig. 8.1a). In addition to the glycolytic pathways, certain carbon sources such as fatty acids and specific amino acids are directly converted to acetyl-CoA and subsequently enter the TCA cycle. Carbon sources of some short chain organic acids such as succinate, malate and citrate can directly enter the TCA cycle through the various points of the pathway during aerobic metabolism (Fig. 8.1a) (Gennis and Stewart 1996). Through these pathways and reactions, various of complex organic carbon sources are fully oxidized and converted to CO_2. This process represents the carbon flow during the aerobic metabolism.

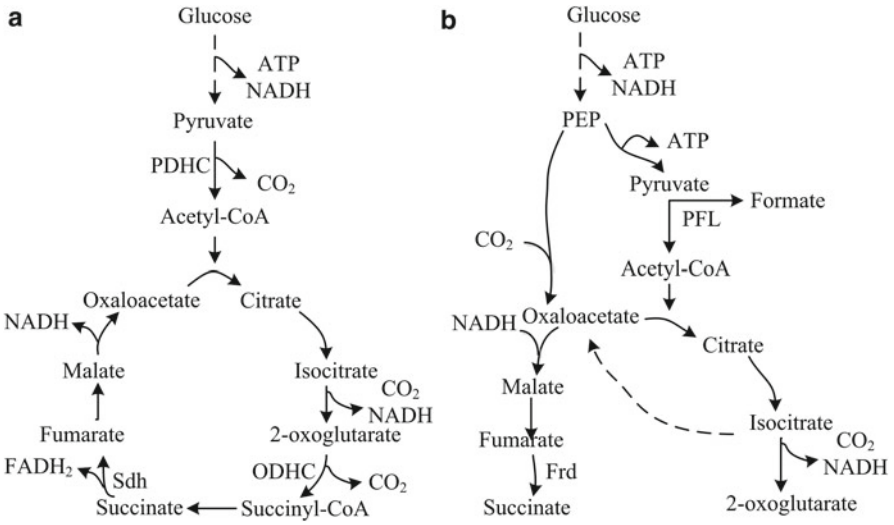

Fig. 8.1 The carbon flow during the aerobic and anaerobic catabolism of glucose. (**a**) Under aerobic condition, glucose is catabolized through glycolytic pathways into pyruvate and subsequently enters the aerobic TCA cycle through which reducing equivalents (in the form of NADH and $FADH_2$) and various carbon intermediates are generated. (**b**) During anaerobic metabolism, the glycolytic pathways are still active, but the TCA cycle becomes branched to generate less NADH. It functions mainly to supply intermediates to biosynthesis under anaerobic conditions. *Broken lines* represent multiple reaction steps. Abbreviations used are: *Frd* fumarate reductase, *ODHC* 2-oxoglutarate dehydrogenase complex, *PFL* pyruvate formate lyase, *PDHC* pyruvate dehydrogenase complex, *PEP* phosphoenolpyruvate, *Sdh* succinate dehydrogenase

Aerobic metabolism also includes the process of electron transport chain which is also the key step for redox homeostasis and energy production. The reducing power in the organic carbon sources is transferred to the electron carrier NADH (with a small portion being transferred to $FADH_2$) through the reactions in the glycolytic pathways and TCA cycle. NADH is the major electron donor during the aerobic respiration and it donates its electrons to the electron transport chain (ETC) located in the cytoplasmic membrane of bacteria. During this process, NADH is oxidized to NAD^+ and electrons released is transferred through a series of electron carriers in ETC and at the end through the terminal reductases to O_2 which is reduced to H_2O following accepting electrons. The electron transport through the ETC generates proton and electrical gradients across the cytoplastic membrane of bacteria which then drives ATP synthesis or be directly utilized to perform other cellular work. In aerobic ETC, the NADH dehydrogenases and the terminal reductases are linked by ubiquinones (Q) as electron carriers (Fig. 8.2a). Depending on the high or low cellular O_2 levels, either cytochrome *o* (Cyo) or cytochrome *d* (Cyd) terminal reductase complex is expressed. While cytochrome *o* complex operates under high oxygen concentration, cytochrome *d* complex is activated and functions under low oxygen concentration such as stationary growth phase owing to its high binding affinity to O_2 but lower activity comparing with the cytochrome *o* complex.

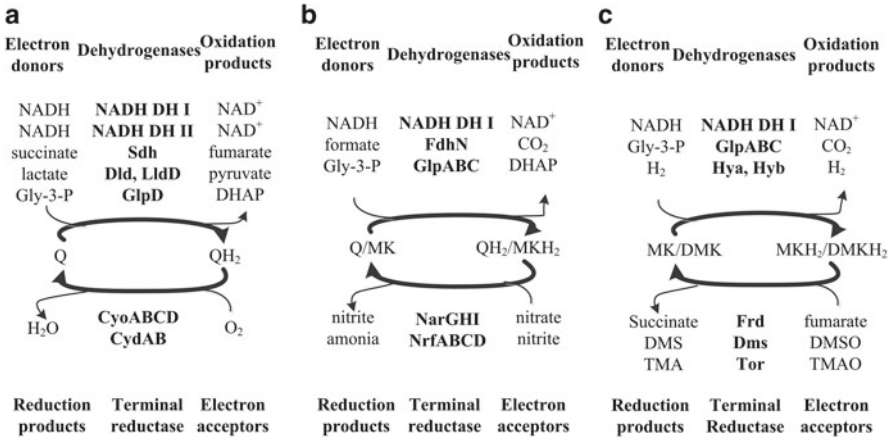

Fig. 8.2 Aerobic or anaerobic electron transport chains (ETC) with different electron carriers, dehydrogenases, and terminal reductases. (**a**) Aerobic electron transport chain. O_2 serves as the terminal electron acceptor of the ETC and either cytochrome *o* or cytochrome *d* complex is the terminal reductase. Ubiquinones serve as electron carriers. NADH, succinate, lactate, or glycerol-3-phosphate can serve as electron donors with the presence of specific dehydrogenases. (**b**) Anaerobic electron transport chain using nitrate or nitrite as electron acceptors. Ubiquinones (UQ) or menaquinone (MK) often serves as electron carriers when nitrate is utilized as the terminal electron acceptor. (**c**) Anaerobic electron transport chain using fumarate, DMSO or TMAO as electron acceptors. Menaquinone (MK) or demethylmenaquinone (DMK) often serves as electron carriers when cells utilize DMSO, TMAO or fumarate as electron acceptors. Abbreviations used are: *CydAB* cytochrome *d* oxidase, *CyoABCD* cytochrome *o* oxidases, *Dld* D-lactate dehydrogenase, *Dms* DMSO reductase, *FdhN* formate dehydrogenase-N, *Frd* fumarate reductase, *GlpD* aerobic glycerol-3-phosphate dehydrogenase, *GlpABC* anaerobic glycerol-3-phosphate dehydrogenase, *Hya* hydrogenase 1, *Hyb* hydrogenase 2, *LldD* L-lactate dehydrogenase, *NADH DH* NADH dehydrogenases, *NarGHI* nitrate reductase A, *NrfABCD* nitrite reductase, *Sdn* succinate dehydrogenase, *Tor* TMAO reductase

Through this ETC coupled ATP synthesis, both energy conservation and redox homeostasis is achieved which is essential to the physiological stability of bacteria.

In addition to NADH, several other compounds can also serve as electron donors to the aerobic ETC which is often linked to the special carbon sources utilized by bacteria. For instance, when glycerol is the sole carbon source available, glycerol-3-phosphate (Gly-3-P), which can be readily generated from glycerol by the enzyme glycerol kinase or derived from degradation of phospholipids and triacylglycerol, is a commonly used electron donor in the ETC (Gennis and Stewart 1996). In this case, glycerol-3-phosphate dehydrogenase (GlpD) is expressed and replaces the NADH dehydrogenase in the ETC which allows glycerol-3-phosphate donates its electrons to the electron carriers in the ETC and be oxidized to form dihydroxyacetone phosphate (DHAP) (Gennis and Stewart 1996). Owing to its less efficiency of energy conservation comparing with that using glucose as the carbon source and NADH as the electron donor, the expression of *glpD* is subject to the control of catabolite repression (Gennis and Stewart 1996). In addition, D-lactate and L-lactate

can also serve as the electron donors of ETC and be oxidized to pyruvate by D-lactate dehydrongenase (Dld) and L-lactate dehydrogenase (LldD) respectively. Some amino acids, such as proline, were also found to be able to serve as electron donors to the ETC in the presence of specific dehydrogenases during the aerobic growth of *E. coli* and *S. typhimurium* (Gennis and Stewart 1996).

8.4 Anaerobic Metabolism of Facultative Anaerobic Bacteria

During aerobic metabolism, the major biological process for redox homeostasis and energy conservation is the cellular respiration using O_2 as the terminal electron acceptor. The large difference of reduction potentials between NADH and O_2 (~1.14 V) ensures the highest yield of energy conservation in bacterial cells. Under anaerobic conditions when O_2 is absent, bacteria must adopt alternative means to oxidize the reducing equivalents NADH and generate ATP as aerobic respiration is no longer operated. Chemoorganotrophs utilize anaerobic respiration or fermentation to support their growth and maintain the cellular redox homeostasis under this condition. Since the compounds which can be utilized to replace O_2 in anaerobic ETC or to oxidize NADH in fermentation all have more positive reduction potentials than O_2, the yield and generation rate of ATP during anaerobic respiration and fermentation is slower than aerobic respiration. As a result of this insufficient oxidizing power, carbon flow is also adjusted under anaerobic growth to maintain redox homeostasis of bacterial cells.

Using glucose catabolism as an example, under anaerobic conditions, it is still converted to pyruvate by glycolysis (Fig. 8.1b). Different from aerobic metabolism, in the next step pyruvate is lysed to yield acetyl-CoA and formate by the pyruvate formate lyase (Pfl) during anaerobic growth. Owing to insufficient oxidizing power under this condition, the TCA cycle which produces majority of NADH during the aerobic metabolism becomes branched and incomplete so that less NADH is produced and the resulting intermediates are mainly used as precursors for biosynthesis. This metabolic reprogramming is achieved by repression of 2-oxoglutarate dehydrogenase complex (ODHC) and replacement of succinate dehydrogenase (Sdh) with fumarate reductase (Frd) in the pathway (Spiro and Guest 1991). Meanwhile, oxaloacetate is replenished from phosphoenolpyruvate (PEP) or isocitrate to maintain the balanced carbon flow in the incomplete TCA cycle (Fig. 8.1b) (Bock and Sawyers 1996; Cornan and Laporte 1996). As a result, less NADH will be generated comparing to aerobic carbon flow in order to cooperate with the lower efficiency of oxidation under this condition (Spiro and Guest 1991).

Under anaerobic conditions, NADH generated during glycolysis and other reactions still must be oxidized to maintain the redox balance and to obtain cellular energy. How NADH is re-oxidized determines which means of anaerobic metabolism, i.e. anaerobic respiration or fermentation, is adopted by chemoorganotrophic bacteria. If bacteria are supplied with exogenous electron acceptors such as nitrate, sulfate, trimethyl-amine-N-oxide (TMAO) *etc.*, they will utilize anaerobic respiration

for energy conservation as ATP generation from the electron transport chain is more efficient than fermentation. If exogenous electron acceptors are not available, bacteria have to utilize endogenous components, i.e. intermediates during catabolic reactions, as electron acceptors to re-oxidize NADH and this process is called fermentation. Environmental factors, such as the availabilities of alternative electron acceptors and fermentable carbon sources, determine which mode of anaerobic metabolism is adopted by bacteria.

8.4.1 Anaerobic Respiration

8.4.1.1 Terminal Electron Acceptors in the Anaerobic Respiration

During anaerobic respiration, the electron donor NADH still transfers its electrons to the quinone electron carriers in the anaerobic ETC via the NADH dehydrogenases complex. Quinones then transfer the electrons to accessible electron acceptors via specific terminal reductases. Different species can utilize different compounds as electron acceptors which often are related to the characteristics of their ecological niches. Common electron acceptors utilized by *E. coli* include nitrate, nitrite, trimethyl-amine-N-oxide (TMAO), dimethyle-sulphoxide (DMSO), and fumarate. Conceivably bacteria prefer electron acceptors with more positive reduction potentials, hence for *E. coli* the order of preferred electron acceptors is oxygen > nitrate > nitrite > DMSO > TMAO > fumarate (Gunsalus and Park 1994). For each electron acceptor utilized in anaerobic respiration, specific terminal reductases are needed (Fig. 8.2b). To achieve optimal energy conservation, the expression of the ETC dehydrogenases and terminal reductases is regulated by both the absence of oxygen and the availability of the corresponding terminal electron acceptors. In *E. coli* under aerobic conditions, the genes involved in the utilization of all other electron acceptors are repressed. Under anaerobic conditions if nitrate is present, DMSO, TMAO, and fumarate respiration is repressed. Only in the absence of oxygen and nitrate, these alternative terminal reductases are expressed (Gennis and Stewart 1996). Different from the aerobic ETC which always utilizes ubiquinones as the electron carriers, anaerobic electron transport chain utilizes different electron carriers depending on the electron acceptors utilized and the presence of corresponding terminal reductases. Instead of ubiquinones, menaquinone (MK) and demethylmenaquinone (DMK) often serves as electron carriers when cells utilize DMSO, TMAO or fumarate as electron acceptors while ubiquinones (UQ) and menaquinone (MK) serves as the electron carriers when nitrate is utilized as the terminal electron acceptor (Unden and Bongaerts 1997). In addition to the common terminal electron acceptors, certain species such as *Geobacter spp.* and *Shewanella oneidensis* can utilize insoluble Fe^{3+} as the terminal electron acceptor (Weber et al. 2006). Some bacteria, especially obligate anaerobes can utilize sulfate and CO_2 as the terminal electron acceptors although they are not common in facultative bacteria.

8.4.1.2 Electron Donors in the Anaerobic Respiration

Similar as in the case of aerobic respiration, some compounds other than NADH can serve as the electron donors to the anaerobic ETC. Examples include several reduced carbon compounds generated from the branched TCA cycle and formate and hydrogen which are generated from pyruvate cleavage by the pyruvate formate lyase. Anaerobic utilization of formate and hydrogen as electron donors requires the expression of formate dehydrogenase (Fdh) and hydrogenase (Hyb) respectively (Gennis and Stewart 1996). Glycerol-3-phosphate can also be utilized as the electron donor under anaerobic conditions with the presence of anaerobic glycerol-3-phosphate dehydrogenase (GlpABC) (Gennis and Stewart 1996). Pyruvate can be oxidized to acetate and carbon dioxide by the pyruvate oxidase (PoxB) and thus can also serve as the electron donor in *E. coli* under anaerobic conditions. However, this pathway is only activated during stationary phase culture and is tightly regulated. The oxidation of pyruvate is suggested to provide as the energy source under microaerobic conditions (Chang et al. 1994).

8.4.1.3 Other Factors Affect the Efficiency of Anaerobic Respiration

In addition to the reduction potentials of different terminal electron acceptors utilized in the anaerobic ETC, presence of other compounds has also found to be essential for the efficiency of anaerobic respiration. One important factor is metal ion homeostasis. This is because many enzymes in the anaerobic respiratory pathways are metal containing enzymes. It was reported that expression of operons containing enzymes in the anaerobic respiration pathway is highly reduced upon iron limitation under anaerobic conditions while the aerobic respiration pathways are only modestly affected in *E. coli* (Cotter et al. 1992). Moreover, induction of DMSO respiration pathways is reported to be dependent on the molybdate uptake (McNicholas et al. 1998).

It is notable that utilization of electron acceptors other than O_2 often produces metabolic intermediates and by-products that differ from aerobic respiration. Some of the intermediates during anaerobic respiration are harmful to bacteria and hence various means to counteract these effects are developed by bacteria when utilize these compounds as electron acceptors. Examples include enzymatic conversion of various nitrosative by-products to catabolizable carbon and nitrogen sources (Rankin et al. 2008) or export them through efflux pumps during the process of nitrate respiration (Zhang et al. 2011). It is also noteworthy that although in most cases the energy conservation mode using nitrate as the electron acceptor occurs only under anaerobic conditions, it may also be adopted under aerobic conditions by some bacterial species. It was reported that *Paracoccus pantothrpus* synthesizes a periplasmic nitrate reductase (NAP) that can conduct aerobic nitrate respiration. NAP is found to be induced under aerobic condition only when highly reduced organic compounds serve as carbon sources. It was proposed that the major purpose of aerobic nitrate respiration is for oxidation of excessive reducing equivalents and to maintain the redox homeostasis of bacterial cells (Sears et al. 2000).

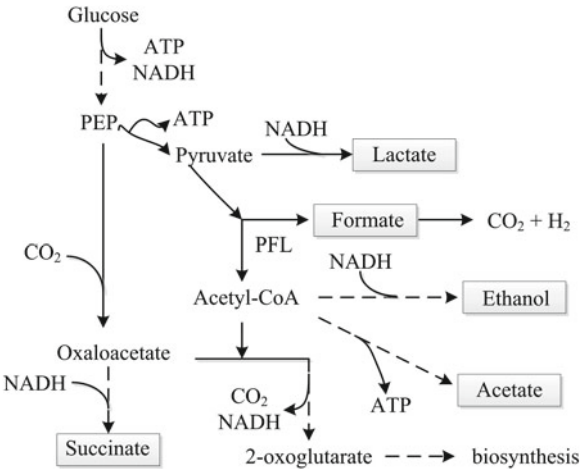

Fig. 8.3 Mixed acid fermentation of glucose by *E. coli*. During anaerobic growth in the absence of electron acceptors, glucose is fermented to form a mixture of products (*boxed*) including ethanol, acetate, lactate, succinate and formate by the model organism *E. coli* in order to regenerated NAD^+ and maintain the redox homeostasis of the cells. Abbreviations used are: *PFL* pyruvate formate lyase, *PEP* phosphoenolpyruvate. *Broken lines* represent multiple reaction steps

8.4.2 Fermentation

Under the conditions that both oxygen and exogenous electron acceptors are not accessible, fermentation will be adopted by facultative anaerobes to maintain their redox homeostasis, during which NADH is oxidized by endogenous intermediates and ATP is only generated by substrate level phosphorylation. Hence, fermentation requires supportable carbon sources that can generate intermediates to be utilized as electron acceptors.

E. coli often conducts a mixed acid fermentation when glucose is the carbon source during anaerobic growth (Fig. 8.3). The final products of mixed acid fermentation include ethanol, acetate, lactate, formate and succinate, which are usually excreted as ethanol, acetic acid, lactic acid, formic acid and succinic acid (Bock and Sawyers 1996). Another common type of fermentation is butanediol fermentation, which is usually adopted by *Enterobacter* (Bock and Sawyers 1996). Different from the aerobic metabolism in which glucose is almost exclusively oxidized to CO_2, the final products of fermentation are diverse and they are largely dependent on the carbon sources utilized and culture conditions. For example, *E. coli* can not use glycerol for fermentation in the absence of other electron acceptors at neutral PH, but under acidic conditions *E. coli* can ferment glycerol and produce ethanol and succinic acid (Dharmadi et al. 2006). In addition, different species may have different capabilities to ferment specific carbon sources. For example, *E. coli* could not grow with citrate as the sole carbon source, whereas *Salmonella typhimurium* can grow with citrate as the carbon source during which energy is derived from citrate fermentation to form acetate, formate and CO_2 (Bott 1997).

Although fermentation has the lowest energy conservation yield among the three metabolic processes, this mode of metabolism has significant biotechnological implications owing to the diversity of the products it can generate depending on the various genetic or environmental factors which provides a robust platform for metabolic engineering. It is notable that accumulation of certain fermentation products may have inhibition effects on cell growth. For example, ethanol, butanol and other alcohols can cause cellular toxicity by interrupting cell membranes (Huffer et al. 2011). Other products such as organic acids affect the pH homeostasis of bacterial cells. In natural microbial communities, these products are often excreted and can be utilized as carbon sources by other species in the same community. However in laboratory or biotechnological growth in a closed vessel, toxic products accumulation in the medium can dramatically inhibit cell growth as well as the yield of desirable products. One common solution to solve this problem is to use chemostat to maintain the physiological state of bacterial cells. Alternatively, genetically modification of bacterial strains to improve their tolerance to target products can be utilized (Ingram 1986) and it represents an active research area in modern microbial biotechnology.

8.5 Regulation of the Switch from Aerobic to Anaerobic Metabolism

8.5.1 Global Transcription Factors FNR and ArcBA

Owing to the significant diversity of anaerobic metabolic pathways and their impact on the global carbon and energy flow in bacterial cells, the transition from the aerobic to anaerobic lifestyle is tightly controlled in facultative bacteria in response to the availability of O_2, electron acceptors, as well as carbon sources. This regulatory network ensures the expression of essential proteins required in specific metabolic pathways, repression of unnecessary pathways, as well as optimal energy conservation under any specific conditions. In *E. coli* the metabolic switch between the aerobic to anaerobic growth is primarily controlled by two global transcription regulatory systems: FNR (fumarate and nitrate reduction) and the ArcBA (aerobic respiratory control) system. Both FNR and AcrA are transcription factors that can bind to specific DNA promoter regions and activate or repress gene transcription (Kiley and Beinert 2003; Gunsalus and Park 1994). The two systems have been found highly conserved among different Gram negative bacterial species, and an FNR-like protein has also been discovered in the Gram positive bacterium *Baccillus subtitlis*. It is interesting that most bacteria which have a FNR-like protein have been found to have a second oxygen or redox responsive protein (Sawers 1999), such as ArcA in the case of *E. coli*, suggesting the necessity of two global regulatory systems to cooperate the metabolic reprogramming in response to O_2 availability.

FNR is a global transcription factor that utilizes an $[4Fe\text{-}4S]^{2+}$ cluster to directly sense and respond to oxygen limitation. Under anaerobic conditions, FNR is synthesized as an $[4Fe\text{-}4S]^{2+}$ cluster containing protein. Assembly of the $[4Fe\text{-}4S]^{2+}$ cluster causes spontaneous FNR dimerization, enabling its specific DNA binding and transcription regulation. Upon being exposed to O_2, the $[4Fe\text{-}4S]^{2+}$ cluster is rapidly oxidized to an $[2Fe\text{-}2S]^{2+}$ cluster which causes the dissociation of FNR dimer and subsequent loss of specific DNA binding and transcription regulation. The O_2 concentration that fully activates FNR is reported to be below 1–5 mbar range of oxygen tensions (Sawers 1999). Upon being activated, FNR activates the expression of enzymes involved in anaerobic respiration and fermentation and represses the expression of those genes in aerobic respiration pathways (Gunsalus and Park 1994). Recent microarray studies reveal that in addition to the genes involved in the carbon and energy flow, FNR regulates more than two hundreds genes in *E. coli* genome and the function of many FNR regulated genes remains unknown (Kang et al. 2005).

While FNR directly senses oxygen through its $[4Fe\text{-}4S]^{2+}$ cluster and regulates gene expression, the ArcBA two component system mainly senses the redox potential in bacterial cells and thus respond to O_2 deprivation in an indirect manner. The system is activated under microaerobic to anaerobic conditions (10–5 mbar oxygen tensions) and functions mainly to repress the enzymes in aerobic metabolic pathways although it also activates genes involved in the microaerobic and anaerobic growth (Sawers 1999). The ArcBA system is a two component system including a membrane sensor protein ArcB and a cytoplasmic response regulator ArcA. Instead of directly sensing O_2 molecules, the transmembrane domain of ArcB senses membrane redox potential, i.e. reduced quinones, and subsequently undergoes phosphorylation at His^{292} and Asp^{576} amino acids at its C-terminal region. The phosphate group is then transferred to ArcA, enabling its capability to bind specific DNA sequences and regulate gene transcription (Rolfe et al. 2011).

Several genes are subject to dual regulation by both FNR and ArcBA. However, the two regulators may have additive or opposite effect on the transcription of the regulated genes. For instance, expression of *pfl* gene, which encodes pyruvate formate lyase that catalyses pyruvate cleavage to acetyl-CoA and formate under anaerobic conditions, is activated by both FNR and ArcBA, whereas the expression of *cydAB* gene which encodes cytochrome *d* oxidase and has optimal expression under microaerobic conditions, is activated by AcrBA but is repressed by FNR with the decrease of oxygen concentration. ArcA is activated first upon the reduction of O_2 level which in turn activates the expression of cytochrome *d* oxidase to optimize cellular respiration under microaerobic conditions. When oxygen concentration is further reduced in the growth environment, FNR will be activated and upon activation it represses the expression of *cydAB*, resulting in the shutdown of aerobic respiration and adoption of anaerobic metabolism under this condition (Tseng et al. 1996). It is conceivable that this cooperative regulation contributes to the fine-tuning of metabolic pathways under a range of different oxygen concentrations (Shalel-Levanon et al. 2005; Rolfe et al. 2011).

8.5.2 *Other Regulatory Factors*

In addition to FNR and ArcBA, NarL and NarP, two regulators that respond to the presence of nitrate and nitrite, often functions cooperatively with FNR and ArcBA and plays an important role in the anaerobic lifestyle of many bacteria. These two regulators activate genes involved in nitrate respiration and repress genes involved in anaerobic respiration of other alternative electron acceptors. Genes regulated by NarL and NarP are simultaneously regulated by FNR in response to the absence of oxygen availability and presence of nitrate. Through cooperative regulation by NarL, NarP and FNR, bacteria can optimize the anaerobic respiration pathways under different environmental conditions such that the carbon and electron acceptors will be utilized in a hierarchical manner to ensure optimal energy conservation under any given conditions (Constantinidou et al. 2006). It is noteworthy that in addition to these global transcription factors, recently a number of small RNAs, such as FnrS and ArcZ, were also identified to participate the regulation of genes involved in the anaerobic switch. The sRNAs FnrS and ArcZ are the components of FNR and ArcA regulon respectively and they function to fine-tune the expression of a subset of genes involved in the anaerobic metabolic reprogramming and enhance the efficiency of the switch (Mandin and Gottesman 2010; Durand and Storz 2010).

It is not unexpected that genetically modification of these transcription regulators and small RNAs can alter the metabolic pathways adopted by bacteria and consequently the metabolic products being generated. Thus it provides a useful tool to engineer the anaerobic metabolic processes. For instance deletion of *fnr* or *arcA* alters the ratio of different fermentation products in *E. coli* (Levanon et al. 2005). Deletion of *arcA* increased ethanol production during glucose fermentation and deletion of both *fnr* and *arcA* caused a high yield of lactate as the major fermentation product presumably due to the inefficiency of pyruvate formate lyase in the mutant strain (Levanon et al. 2005). These approaches will be further discussed in the following section.

8.6 Application and Manipulation of Anaerobic Metabolic Reprogramming in Biotechnology

Comparing with aerobic metabolism, anaerobic metabolism especially fermentation can lead to the production of a broad range of valuable chemicals which have great biotechnological implications. To apply and manipulate bacterial metabolic pathways towards the maximal production of the desirable products, the fundamental principles of maintaining the redox and energy homeostasis of bacteria must be fulfilled. On the basis of these fundamental principles, several environment based, process based, as well as strain based approaches can be applied to manipulate and engineer the metabolic pathways. Environment based approaches include control of environmental factors, such as the availability of carbon, nitrogen, and electron acceptors, as well as other factors such as pH, pressure, temperature *etc*. Process based

approaches refer to those that alter the mechanical and physical properties of the reaction systems, and strain based approaches include genetically engineering bacterial strains to overexpress target enzymes or block pathways competing for the same substrate. In this section we use glycerol fermentation as a paradigm to illustrate these strategies and applications.

8.6.1 Fermentative Metabolism of Glycerol

Fermentation of sugars to generate various chemicals such as ethanol, CO_2, and lactic acid has widely been utilized in industrial applications such as alcoholic beverage, food, and biofuels. The fermentative conversion of 6-carbon sugars derived from corn, sugarcane or sugar beet into the transportation biofuel ethanol has especially represented a major biotechnological development in the last decade owing to its potential to replace the fossil fuels. However, a major problem that hampers its economic viability is the co-production of a large amount of glycerol, which has resulted in a dramatic surplus of this by-product and a more than ten-fold decrease of the price of crude glycerol (Yazdani and Gonzalez 2007). How to convert this low price by-product to higher value products has become both an urgent need and a "target of opportunity" to increase the economic viability of biofuels industry (Yazdani and Gonzalez 2007).

Compare with glucose, glycerol is a carbon source with a higher degree of reduction. Several species of the *Enterobacteriaceae* family, such as *Klebsiella*, *Citrobacter*, and *Enterobacter*, and the genera of *Clostridium*, *Lactobacillus*, and *Bacillus* which contain the enzyme of 1,3-propanediol dehydrogenase (1,3-PDODH), can ferment glycerol to produce 1,3-propanediol (Yazdani and Gonzalez 2007). *E. coli*, the workhorse of modern biotechnology, was considered to be unable to ferment glycerol owing to its high reduction state. However, Dharmadi et al. (2006) have studied the feasibility of *E. coli* K-12 strain to ferment glycerol and found that *E. coli* MG1655 is able to ferment glycerol anaerobically in a pH dependent manner and the reaction requires the enzyme formate hydrogen lyase (FHL) (Fig. 8.4). Using different bacterial species and through metabolic engineering, glycerol fermentation can be utilized to produce several valuable chemicals, such as 1,3-propanediol, ethanol, succinic acid, propionic acid, and dihydroxyacetone *etc*.(da Silva et al. 2009). We next summarize the various strategies utilized to promote the production of each of these chemicals through metabolic engineering of the glycerol fermentation pathways.

8.6.2 1,3-Propanediol

Production of 1,3-propanediol (1,3-PDO) has attracted considerable interest in the past several years due to its promising applications in cosmetic and polymer industries (Saxena et al. 2009). Fermentative production of 1,3-PDO by bacterial species such as *Klebsiella* and *Citrobacter* requires the enzyme 1,3-PDODH to convert 3-hydroxypropionaldehyde, the dehydrating product of glycerol, to the 1,3-PDO

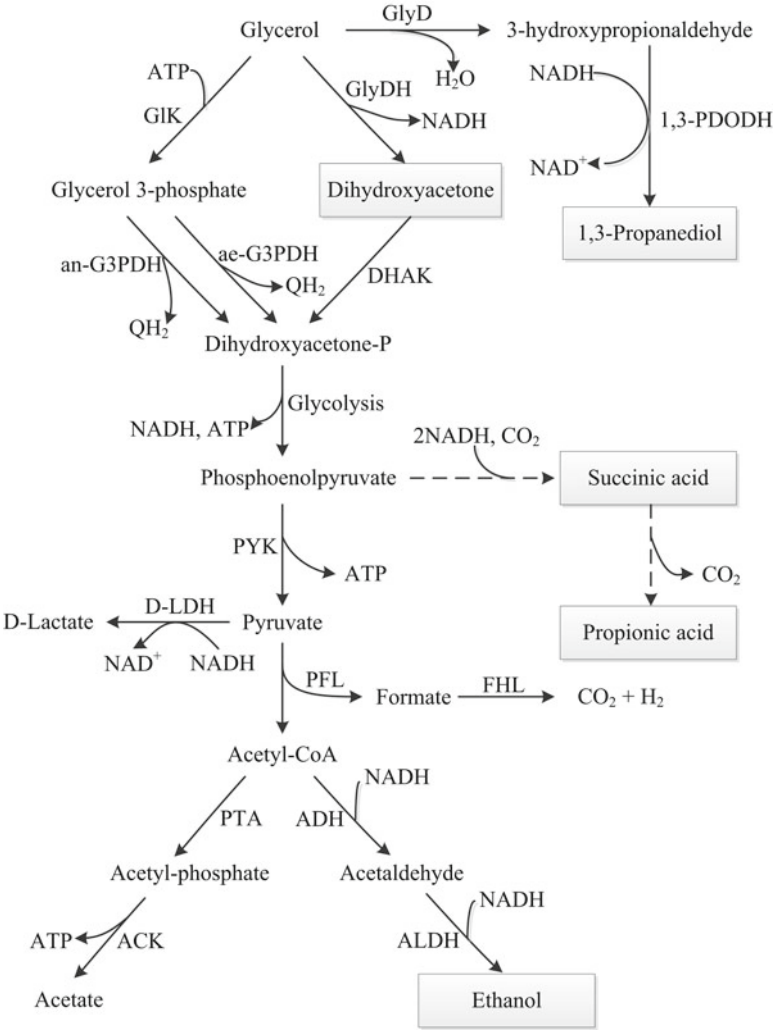

Fig. 8.4 Glycerol dissimilation and the synthesis of metabolites in bacteria under anaerobic and microaerobic conditions. In the species of *Klebsiella*, *Citrobacter*, *Enterobacter*, *Clostridium*, *Lactobacillus*, and *Bacillus,* glycerol fermentation can be divided into oxidative and reductive pathways. In the oxidative pathway, glycerol is converted to glycolytic intermediate dihydroxyacetone-P which is then degraded to diverse metabolites with the production of reducing equivalents. The reductive pathway, represented by the production of 1,3-propanediol, acts as a sink for the reducing equivalents generated in the oxidative pathway to maintain the redox balance during the metabolism. Important metabolites are boxed. *Broken lines* represent multiple reaction steps. Abbreviations used are: *ACK* acetate kinase, *ADH* alcohol dehydrogenase, *ALDH* aldehyde dehydrogenase, *ae-G3PDH* aerobic glycerol-3-phosphate dehydrogenase, *an-G3PDH* anaerobic glycerol-3-phosphate dehydrogenase, *DHAK* dihydroxyacetone kinase, *FHL* formate hydrogen-lyase, *GlK* glycerol kinase, *GlyD* glycerol dehydratase, *GlyDH* glycerol dehydrogenase, *LDH* lactate dehydrogenase, *PFL* pyruvate formate-lyase, *PTA* phosphoacetyl transferase, *PYK* pyruvate kinase; *1,3-PDODH* 1,3-propanediol dehydrogenase

product (Fig. 8.4). This step is essential and is referred as the reductive pathway of the process as it consumes NADH generated during glycolysis of glycerol to pyruvate (referred as the oxidative pathway of the process) and regenerates NAD^+. Both process based and strain based strategies were developed to optimize the production of 1,3-PDO. For example, a major problem of using glycerol as the sole carbon source is the slow growth and low biomass in the fermentor. To overcome this, a two-stage fermentation process was developed by Boenigk et al. (1993) in which sufficient biomass was produced in the first fermentor under low glycerol concentration (250 mM) but supplemented with 0.02% yeast extract, and production of 1,3-PDO was initiated and significantly increased by the addition of glycerol (625 mM) in the second fermentor following the accumulation of the biomass. The second approach to improve cell growth involves changing the anaerobic condition to microaerobic conditions (Chen et al. 2003). This modification increases the productivity of 1,3-PDO from 0.8 to 1.57 $g\,l^{-1}\,h^{-1}$. Improving cell growth can also be achieved by supplement of fumarate (5 mM), an electron acceptor for anaerobic respiration, to bacterial cultures such as *Klebsiella pneumoniae*. Through this approach, both the rate of glycerol consumption and 1,3-PDO production increased 35% compared with that in the absence of fumarate (Lin et al. 2005). This is presumably because fumarate can enhance the activities of several key enzymes in the pathway and decreases the NAD^+/NADH ratio resulting in more available reducing power to convert 3-HPA to 1,3-PDO. Production of 1,3-PDO can also be improved through strain based approaches. For example, to eliminate ethanol, a major competitor in the production of 1,3-PDO, aldehyde dehydrogenase gene (*aldA*) which plays a significant role in ethanol formation in *Klebsiella pneumoniae* was deleted and this enhanced the productivity and yield of 1,3-PDO to 14.05 $mmol\,L^{-1}\,h^{-1}$ and 0.699 $mol\,mol^{-1}$ respectively (Zhang et al. 2006). To eliminate the toxic effect of organic acid byproduct lactate, Yang *et. al* deleted lactate dehydrogenase gene (*ldhA*) of *Klebsiella oxytoca* and found that gene deletion led to 58 and 21% increase of the concentration and productivity of 1,3-PDO respectively grown under microaerobic conditions (Yang et al. 2007). Finally, it was reported that using *Clostridium butyricum* VPI 3266, the best natural 1,3-PDO producer identified so far, the productivity of 1,3-PDO was achieved to the highest level (10.3 $g\,l^{-1}\,h^{-1}$) at dilution rate of 0.30 h^{-1} and 60 $g\,l^{-1}$ of feed glycerol fermentation pattern (Gonzalez-Pajuelo et al. 2005).

8.6.3 Ethanol

Dharmadi et al. (2006) studied glycerol fermentation by *E. coli* at acidic pH and found that ethanol accounted for 86% of all products in the fermentation broth. A small percentage of succinic acid (7%) was also identified, this is because both ethanol and succinic acid production through glycerol fermentation is redox-balanced process (Fig. 8.4). However, compare with succinic acid production pathway, the reactions led to ethanol production have a net gain of one ATP per each molecule of

glycerol being converted, thus glycerol fermentation by *E. coli* is promising to be utilized to produce ethanol. Several means have been found to be effective to further improve the yield of ethanol production. These include using high concentration of glycerol to overcome the low affinity of glycerol dehydrogenase to glycerol substrate (Gonzalez et al. 2008); using microaerobic conditions to replace strict anaerobic conditions which greatly improve bacterial growth and eliminate the need for rich supplements such as tryptone or yeast extract (Durnin et al. 2009); as well as lower the concentration of potassium and phosphate which has a negative effect on the redox balance of the fermentation process (Gonzalez et al. 2008). Furthermore, *Paenibacillus macerans*, a species in the genus of *Paenibacillus*, which has broader metabolic capabilities than *E. coli* was exploited to ferment glycerol by Gupta et al. (2009) and it was found that the maximum specific growth rate of *P. macerans* during the log phase can achieve $0.40 \pm 0.03\ h^{-1}$, about ten-fold of that reported for *E. coli,* and the rate of ethanol production was 12 fold higher than that in *E. coli*.

8.6.4 Succinic Acid

Succinic acid is a biorefinery that has broad industrial values. Owing to its reduced state, succinic acid production from glucose fermentation has been limited (Sawers and Clark 2004), whereas fermentation of glycerol, a carbon source with higher degree of reduction, provides a promising means for its production (Fig. 8.4) (Dharmadi et al. 2006). Although glycerol fermentation by *E. coli* led to only ~7% succinic acid, its production has been found can be dramatically increased to 22% by simply conducting the fermentation in the presence of CO_2 (Dharmadi et al. 2006). Another species that can ferment glycerol to produce succinic acid is *Anaerobiospirillum succiniciproducens* and fermentation of glycerol by *A. succiniciproducens* led to a much higher yield of succinic acid (133%, g-succinic acid/g-glycerol) than fermentation of glucose (86%) (Lee et al. 2001). Cell growth, succinic acid yield, and productivity were found to be significantly enhanced when the culturing system of *A. succiniciproducens* was supplemented with hydrogen as an external electron donor in the form of H_2/CO_2 mixture (5:95, v/v) (Lee et al. 1999). Supplement with specific components have also found to be effective to increase succinic acid production by *Enterococcus flavescens* through glycerol fermentation. It was reported that a seven-fold increase of productivity was achieved when the culture medium was supplied with 3% sucrose, 15 mM $MgCO_3$, 1:0.5 ratio of tryptone and ammonium hydrogen phosphate at pH 6.5, and incubated at 39 °C (Agarwal et al. 2007).

8.6.5 Propionic Acid

Comparing with glucose, glycerol is a more optimal carbon source for propionic acid production owing to its low price, sufficient availability, higher yield, and reduced cost of purification (Yazdani and Gonzalez 2007; Barbirato et al. 1997). It was reported that production of propionic acid can be improved by co-fermentation of glycerol

(mainly used for propionic acid production) and glucose (provide both reducing equivalents and ATP for biomass) by the species of *Propionibacterium acidipropionici* (Liu et al. 2011). Comparing with the process using glycerol as the sole carbon source, a mixture of glycerol/glucose with 4/1 (mol/mol) ratio has led to 20 and 21% increase of the yield and productivity of propionic acid respectively. Feeding glycerol at a constant rate (fed-batch fermentation) provides another means to improve both the yield and productivity of propionic acid since this can help to eliminate the cell growth inhibition caused by propionic acid (Liang et al. 2012; Feng et al. 2010; Zhu et al. 2010). Strain based approach was also investigated to improve the yield of propionic acid. During glycerol fermentation by *Propionibacterium acidipropionici*, it was found that deletion of acetate kinase gene which eliminates the formation of competitive product acetate can increase the concentration of propionic acid in fed-batch fermentation broth to 100 $g\,l^{-1}$, which is much higher than that produced by the wild-type strain (Zhang and Yang 2009).

8.6.6 Dihydroxyacetone

Glycerol fermentation can also be utilized to produce dihydroxyacetone, an important intermediate for various chemicals in industry (Fig. 8.4). Two approaches have been investigated that can increase the productivity of dihydroxyacetone from glycerol fermentation. A strain based optimization revealed that overexpression of glycerol dehydrogenase gene can improve both bacterial growth and production of dihydroxyacetone by *Gluconobacter oxydans* (Gätgens et al. 2007). In addition, process based approach such as using repeated-fed-batch mode of fermentation, which eliminates the inhibition of both the substrates and products on bacterial growth, caused 75% increase of the productivity of dihydroxyacetone (Hekmat et al. 2003).

8.7 Concluding Remarks

It is noteworthy that in addition to the glucose and glycerol fermentation mentioned here, microbial anaerobic metabolism has much broader applications in various areas of industry, environment and medicine. Examples include bio-hydrogen production by the photosynthetic non-sulfur bacteria (PNSB) which eventually convert solar energy to usable hydrogen fuels (McKinlay and Harwood 2010; Argun and Kargi 2011; Basak and Das 2007; Yokoi et al. 2002; Asada et al. 2006), application of anaerobic sulfate reduction to remove heavy metal contamination (Muyzer and Stams 2008) and anaerobic ammonium oxidation (anammox) to promote the global nitrogen cycle (van der Star et al. 2007), as well as the recent exploitation of hypoxia targeted bacterial strains *Salmonella*, *Clostridium*, and *Bifidobacterium* for cancer treatment (Arrach et al. 2008; Forbes 2010; Ryan et al. 2009). While designing and manipulation of these systems must fulfill the fundamental principles of

energy and redox homeostasis of bacterial physiology, optimizing various environmental, process, and strain based factors can be explicitly determined by experiments. There is no doubt that with our increasing understanding of the physiology and biochemistry of microorganisms, identification and sequencing of more bacterial species, as well as the development of system-wide approaches, exploitation of microbial metabolism will have greater contribution to many areas of human life and have significant impact on human heath in the future.

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