Microbiology, Vol. 69, No. 2, 2000, pp. 119–128. Translated from Mikrobiologiya, Vol. 69, No. 2, 2000, pp. 149–162.

REVIEW PAPERS

Physiology of Microbial Cells and Metabolic Engineering

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Received March 2, 1999; in final form, October 7, 1999

Abstract—This review is devoted to the problems of the physiology and cell biology of microorganisms in relation to metabolic engineering. The latter is considered as a branch of fundamental and applied biotechnology aimed at controlling microbial metabolism by methods of genetic engineering and classical genetics and based on intimate knowledge of cell metabolism. Attention is also given to the problems associated with the metabolic limitation of microbial biosyntheses, analysis and control of metabolic fluxes, rigidity of metabolic pathways, the role of pleiotropic (global) regulatory systems in the control of metabolic fluxes, and prospects of physiological and evolutionary approaches in metabolic engineering.

Key words: metabolic engineering, analysis of metabolic fluxes, physiology of microbial cells

Bailey was the first to acknowledge, in the early 1990s, metabolic engineering as a new science and independent line of research [1]. Formally, this science is very close to genetic engineering, the more so that the latter is a key methodological approach in metabolic engineering. There are different definitions of metabolic engineering [2], each of which emphasizes particular aspects of this science. The term *metabolic engineering*, which is obviously derived from the terms *metabolism* and *genetic engineering*, highlights the fact that the improvement of metabolic pathways by controlling the respective enzymes, transport proteins, and regulatory molecules with the use of the recombinant DNA approach is possible only on the bases of intimate knowledge of microbial metabolism and its regulation.

Proceeding from this basic Bailey's definition, we arrive at the following definition of metabolic engineering as a science. "Metabolic engineering is a scientific approach aimed at the control of metabolic pathways via directed changes in genomes primarily by methods of genetic engineering and classical genetics." Undoubtedly, metabolic engineering extensively uses the research tools of protein engineering, experimental evolution, genetic selection, microbial cultivation, etc. The engineering of macromolecules is a special problem, which is beyond the scope of the present review. However, some examples of the application of physiological approaches in metabolic engineering will be considered below.

A number of recent reviews [2–8] give numerous examples of how metabolic engineering methods can be efficiently used to enhance the level and variety of microbially produced compounds, to widen the range of utilizable substrates, to modify metabolic pathways and create new ones (for instance, for the degradation of xenobiotics), and so on. Analysis of information presented in these reviews and in numerous relevant experimental articles allows the inference to be made that further development of metabolic engineering is hardly possible without intimate knowledge of microbial metabolism, especially primary and intermediary metabolism. It should be emphasized that the metabolic engineering of primary and intermediary metabolism is especially difficult because of their complex and multilevel regulation.

It is these problems that we consider in our review, placing special emphasis on the metabolic limitation of microbial syntheses, the rigidity of the metabolism of host cells, the role of global (pleiotropic) regulatory systems in the expression of recombinant phenotypes, and on the feasibility of physiological approaches in metabolic engineering.

METABOLIC LIMITATION OF CELLULAR PROCESSES

The concept of metabolic limitation. One of the primary methodological approaches in metabolic engineering aimed at intensifying the activity of particular enzymes, metabolic pathways, and microbial metabolism as a whole lies in the compensation of metabolic bottlenecks, or metabolic limitation sites [9]. In a general form, the concept of metabolic bottlenecks has been formulated by Monod [10]. The relevant literature is now fairly extensive (for key publications, see [11–18]).

As a matter of fact, we may speak about several levels of metabolic limitation. Limitation at the level of particular enzymes is due to their low activity or suppression under the action of negative regulatory circuits. The degree and the location of metabolic limitation are not constant and depend on the cultivation conditions, growth substrate, and growth rate in particular

0026-2617/00/6902-0119$25.00

[16, 17]. Generally, however, metabolic limitation is due to the suppression of several or even all components of a metabolic pathway [19, 20].

Limitation can also occur at the level of a macromolecular complex or metabolic system, such as the apparatuses responsible for protein synthesis, chromosome replication, and energy generation [11, 13, 15]. It remains unclear whether such limitation is associated with the whole system functioning as a single metabolic element [21] or is due to a local limitation site within the system. For instance, based on the results of the numerical simulation of *Saccharomyces cerevisiae* respiration, which is considered to be the limiting factor of the aerobic growth of this yeast, Barford [22] assumed that the actual site of limitation is the transport of tricarboxylic acids to mitochondria. On the other hand, glycolytic flux in yeasts was found to be limited by glucose transport to cells or by the activity of inducible catabolic enzymes, such as phosphofructokinase [18, 23].

Protein synthesis can be limited by the number of free ribosomes in bacterial cells [24] or by the ADP : ATP and GDP : GTP concentration ratios in mammalian cells (in these experiments, the efficiency of protein synthesis was estimated in vitro) [25]. Designing specialized ribosomes for the synthesis of certain proteins showed that protein synthesis may also be limited at the level of mRNA binding to ribosomes. Such limitation of protein synthesis can be prevented by inducing some mutations in the anti-Shine–Dalgarno-like sequences of 16S rRNA. The initiation of translation can also be enhanced through the creation of hybrid proteins and operons [26, 27].

In the case of highly integrated macromolecular complexes or metabolic systems, such as the protein-synthesizing apparatus, some genes of which are expressed under negative autogenous control at the level of transcription and/or translation [28], it is very difficult to properly identify the actual site of metabolic limitation and especially to compensate for it by genetic engineering facilities.

Identification of metabolic limitation sites is a prerequisite for the successful solution of many problems of metabolic engineering related to the intensification of the production of particular metabolites or biomass. When metabolic engineering was in its infancy, metabolic bottlenecks were usually identified based on the general knowledge of cell metabolism and its regulation.

Such an approach, however, sometimes led to erroneous results. An example of this is the attempt of Windass *et al.* [29] to improve the conversion of ethanol to single-cell protein by cloning glutamate dehydrogenase from *Escherichia coli* into the methylotrophic bacterium *Methylophilus methylotrophus*. The authors believed that the substitution of glutamate dehydrogenase for glutamine synthetase in *M. methylotrophus* cells should enhance the conversion of methanol into biomass, since glutamate dehydrogenase does not need ATP and, hence, is energetically more favorable than glutamine synthetase.

Actually, however, glutamate dehydrogenase does need NADPH in the reaction of ammonium assimilation and, therefore, cannot be considered to be energetically more favorable than the ATP-dependent glutamine synthetase. Moreover, the higher K_m of glutamate dehydrogenase with respect to ammonium in comparison with the glutamine synthetase–glutamate synthase system and the stringent control of the energetic status of cells make the idea of Windass *et all.* still more questionable. Indeed, the recombinant strain accumulated biomass that was only 4–7% higher than that of the wild-type strain, which is within the limits of experimental error.

The approach based on establishing a correlation between some metabolic parameters can also give ambiguous results. Thus, the correlation derived by Herbert and Kornberg between the activity of the phosphoenolpyruvate-dependent phosphotransferase system (PTS) of glucose transport and the growth rate of *E. coli* allowed them to infer that bacterial growth is limited by the activity of PTS [12]. This inference, however, is valid only for low concentrations of glucose, since, at higher glucose concentrations, other transport systems, including nonspecific ones, come into operation [30].

Among alternative approaches aimed at identifying metabolic bottlenecks, we should first of all mention the theory of metabolic flux control (TMFC), which was proposed by Kacser and Burns [19] and Heinrich and Rapoport [20]. This theory is based on the assumption that enzymes in a metabolic pathway are differently saturated with their substrates and, hence, can differently affect metabolic flux via this pathway. The knowledge of the catalytic characteristics of the enzymes and the concentrations of the intermediates of the pathway makes it possible to estimate the contribution of each of the enzymes to the control of metabolic flux. This contribution can be described by two coefficients, the coefficient of metabolic flux control, which is the ratio of the change in the activity of an enzyme to the change in the metabolic flux through it, and the coefficient of enzyme elasticity (sensitivity), which is the ratio of the change in the concentration of the enzyme substrate to the change in its activity (one of the latest reviews on this topic is [31]). The sum of the control coefficients of enzymes of a given metabolic pathway is equal to 1 (or 100%). The problem is reduced to finding the enzyme with the maximum control coefficient. In practice, control coefficients rarely exceed a value of 0.5; however, frequently encountered values are still lower, which indicates that the controlling capacities of enzymes do not greatly differ.

The TMFC has been from the outset a subject of criticism. First, this theory considers only linear enzyme sequences, while it is the branching sites that

exert the most stringent regulation of metabolic fluxes. Second, the theory suggests that enzymes obey the Michaelis–Menten kinetics, while there is increasing evidence that enzymes, even in prokaryotic cells, are organized in complexes which in turn form an enzoskeleton [33]. As a result, enzymes interact with each other and, hence, do not obey the Michaelis–Menten kinetics, which is valid only for individual enzymes occurring in a solution. Third, the theory, while postulating the existence of rate-limiting and controlled enzymes, virtually does not take into account the allosteric regulation of enzymes by coenzymes, energy-rich compounds, metabolites, and the effect of pleiotropic (global) regulatory systems [32–34]. All this often leads to the failure of attempts to identify metabolic sites appropriate to control metabolic fluxes [35].

The alternative theory of metabolic flux analysis (TMFA), which was proposed by Crabtree and Newsholm [36] and is now being developed by Holms [37], is based on the idea of measuring metabolic fluxes in cells through the analysis of the balance of substrates, end products, and intermediates. Using a chemostat culture growing at a constant rate, one calculates the yield of biomass and extracellular metabolites and the intracellular concentrations of all intermediates in a metabolic pathway or cellular compartment with respect to the growth rate. Analysis in terms of this theory can be supported by the thermodynamic analysis of enzyme reactions. The data thus obtained characterize metabolic fluxes through each enzyme; this allows a metabolic bottleneck to be easily determined. It can be seen that the theory under discussion obviates the need to know the kinetic properties of enzymes.

Metabolic fluxes can also be analyzed in situ using labeled substrates and nuclear magnetic resonance [6, 38]. In spite of some disadvantages associated with the necessity of working with very dense cell suspensions, this method has proved to be rather efficient [6, 38].

Among other methods of metabolic flux analysis, we should mention those based on the stoichiometry of the intermediates of a metabolic pathway [35], the dynamics of intracellular pools of metabolic intermediates in response to the fractionated addition of certain substrates to stationary-phase bacterial cultures [39], titration of enzymes with specific inhibitors, the dynamics of culture growth on various substrates [40], as well as methods combining these approaches [41]. Mathematical modeling has also been used to identify the metabolic limitation sites [42]. As for the merits and demerits of these methods, they are well characterized by Brown: "All models are invalid, although some of them are useful" [43].

BOTTLENECK COMPENSATION AND METABOLIC RIGIDITY

Methods of classical genetics and genetic selection allow some problems of metabolic engineering to be solved: to change metabolic fluxes in cells in such a way as to provide the enzyme responsible for synthesis of a pertinent product with the maximum amount of substrate; to minimize the efflux of intermediates; and to suppress negative regulatory circuits and the conversion of the pertinent product into unwanted by-products. The potentiality of these methods is, however, restricted to the genotype of the producing microorganism. Therefore, methods of classical genetics are less potent than those of genetic engineering.

Physiological approaches in metabolic engineering are still less potent than methods of classical genetics. However, some particular problems of metabolic engineering can be solved with the use of physiological approaches. For example, continuous cultivation with regular changes in the growth rate from the maximum to the minimum and back was successfully employed to maximize the DNA polymerase I yield in recombinant strains [44].

Another example of physiological approaches in metabolic engineering is the use of the phenomenon of cometabolism for the biotransformation of some compounds [45–47]. However, these works were carried out without changing the phenotype and, hence, can hardly be considered as belonging to metabolic engineering.

More representative in this respect is the work of Novick and Horiuchi [48], who obtained a hyperproducer of β-galactosidase through selection of the genetic variants of the *E. coli* strain subjected to severe selective pressure in a chemostat culture. Actually, this work has laid the foundation for a promising new trend in biotechnology: evolutionary biotechnology.

Nevertheless, genetic engineering remains the main tool for the compensation of metabolic limitations in microbial producers.

Metabolic rigidity. Stephanopoulos and Vallino [49] defined the rigidity of the metabolic network as its inherent resistance to changes in metabolic fluxes. In their work, they concentrated on studying the regulatory mechanisms responsible for the distribution and stabilization of metabolic fluxes at branching points, or metabolic nodes. It is clear that metabolic rigidity can be due to several factors: (1) the absence of distinct bottlenecks in metabolic pathways; (2) the multilevel negative feedback regulation ranging from local regulatory mechanisms that function in the limits of a metabolic pathway to global (pleiotropic) mechanisms, (3) the doubling of regulatory mechanisms; and (4) the high degree of the integrity and hierarchy of regulatory systems in bacterial cells.

The different rigidity of metabolic blocks in microorganisms can be easily seen from the comparison of glycolysis and the tricarboxylic acid (TCA) cycle in the

facultative anaerobes *E. coli* and *S. cerevisiae*. In attempts to elucidate the physiological role of the TCA cycle in *E. coli*, Lee *et al.* [50] studied the energetics of its citrate synthase–deficient gltA mutant and found that blocking the glycolytic flux at the level of its entrance into the TCA cycle gives rise to ATP production in amounts comparable to those observed in the wild-type *E. coli* strain. Paradoxically, the role of the TCA cycle in *E. coli* is mainly biosynthetic, while ATP is primarily produced in glycolysis, both anaerobic and aerobic.

It should be noted that these data can be interpreted alternatively. A mutant with a blocked TCA cycle is physiologically identical to the wild-type strain under anaerobic conditions, when the glycolytic flux increases severalfold (this phenomenon is known as the Pasteur effect) and thus compensates for the deficiency of energy resulting from the TCA cycle blockade. An analogous response to a rise in the concentration of glucose in the medium is known as the Crabstree effect. All this suggests that the high plasticity of glycolysis is due to the fact that facultative anaerobes must have a mechanism that compensates for the blocked TCA cycle through the enhancement of substrate-level phosphorylation in glycolysis. In turn, the increased glycolytic flux under anaerobic conditions necessitates the existence of mechanisms for dumping excessive glycolytic products (such products, primarily acetate, are usually excreted into the medium). The mechanism of the Crabstree effect is analogous; i.e., the activation of glycolysis by high concentrations of glucose leads to an excess of glycolytic products with respective consequences [51]. Changes in the rate of glycolysis, which are typical of facultative anaerobes, are also due to its manifold role, namely, glycolysis provides cells with energy and biosynthetic processes with necessary intermediates. This explains the complex character of the regulation of glycolysis and the existence of distinct glycolytic bottlenecks.

Bottleneck compensation in intermediary and central metabolism. The aforementioned peculiarities of facultative anaerobes offer good possibilities for manipulating with glycolytic flux under aerobic conditions, when its level is far from maximum. For these reasons, the examples of successful bottleneck compensation in both the central glycolytic pathway and its shunts are numerous.

The central metabolism and energetic status of cells are stringently controlled by homeostatic mechanisms. This explains why a deletion in the membrane ATP synthase gene in *E. coli* caused only an insignificant decrease in the growth rate and biomass of this bacterium [52]. Under aerobic conditions, metabolic homeostasis is responsible for the severe rigidity of the TCA cycle. For instance, the genetically enhanced expression of the *E. coli* genes controlling the TCA cycle brought about a metabolic imbalance and decreased the growth rate of this bacterium [53]. Similarly, the amplification of the fumarate reductase gene in *E. coli* led to the accumulation of succinate and/or intracellular lipid–protein granules but did not influence bacterial growth [54, 55].

The role of enzymatic bottlenecks in facultative anaerobes at the level of the glycolytic flux entrance into the TCA and glyoxylate cycles lies in the partitioning of metabolic fluxes to various biosynthetic pathways. This was confirmed by genetic engineering methods. For example, the amplification of the PEP carboxylase gene enhanced threonine synthesis in *Serratia marcescens* and *Brevibacterium lactofermentum* [56, 57] and led to succinate accumulation in the wild-type *E. coli* strain [58]. The creation of a futile cycle between phosphoenolpyruvate and oxalacetate by the concurrent amplification of the PEP carboxykinase and PEP carboxylase genes stimulated glycolytic flux, which manifested itself as an enhanced consumption of glucose and oxygen [59]. Cloning of the highly active pyruvate decarboxylase gene from *Zymomonas mobilis* to *E. coli* considerably augmented ethanol production and decreased the generation of unwanted fermentation products [60]. The same operation with *Klebsiella planticola* improved the production of ethanol from xylose [61]; the inactivation of the pyruvate formate-lyase gene enhanced ethanol production still further [62].

THE ROLE OF GLOBAL REGULATORY SYSTEMS

Concentrating on the rate-limiting enzymes of metabolic fluxes, researchers often neglect the regulatory systems of other levels, such as the global regulatory system. Meanwhile, this may lead to a misinterpretation of the experimental data. This situation can be well exemplified by the many-year discussion concerning the question "Is phosphofructokinase a rate-limiting enzyme in eukaryotic cells?" To answer this question correctly, it is necessary to consider the regulation of yeast glycolysis by invoking recent data.

Regulation of glycolysis in *S. cerevisiae*. In terms of the TMFC, the coefficients of metabolic flux control for phosphofructokinase and hexokinase are 0.3 and 0.7, respectively. Such high values of these coefficients imply that the contribution of these enzymes, especially hexokinase, to the regulation of glycolysis is great [63]. Some researchers questioned the validity of the TMFC, emphasizing that the effect of cofactors, compartmentation, and enzyme interactions can change the K_m of glycolytic enzymes by an order of magnitude [64]. In eukaryotic cells, phosphofructokinase, which is under the stringent control of energy-rich intermediates, is prone to oscillatory behavior (such as ultimate cycling) and multiple steady states of the hysteresis type with spontaneous transitions between them [65]. In view of this, some authors consider phosphoglycerate kinase, another glycolytic enzyme, to be the most likely candidate for the role of the rate-limiting enzyme of glycolysis [39, 64]. On the other hand, the dynamics of some

components of the biomass of *Rhodosporidium toruloides*, a superproducer of intracellular lipids, implies that it is phosphofructokinase that controls lipid synthesis in this yeast [18]. It should, however, be noted that, unlike *S. cerevisiae* phosphofructokinase, *R. toruloides* phosphofructokinase is not under the stringent control of energy-rich adenylates.

To illustrate the complicacy of the problem of identification of rate-limiting steps in yeast and fungal glycolysis, let us briefly consider glycolytic regulation.

The primary negative regulatory signal is generated by glucose already at the level of glucose reception by transport proteins [66, 67]. The transport proteins (they are 20 in number, but only seven of them, Hxt1–Hxt7, are essential for the normal functioning of cells) respond to various concentrations of glucose by changing their K_m values [68, 69]. This process involves the regulator protein Snf3 responsible for a decrease in K_m at low glucose concentrations, the regulator protein Rgt2 with the opposite function, Snf1 protein, and the general glucose sensor protein Ggs1 [66, 67]. The decrease in K_m may be due to the removal of the suppressing effect of high concentrations of glucose on a high-affinity glucose transporter or, which is more likely, on the regulator protein Snf3. A mutant with a damaged adenylate cyclase gene (CDC35) exhibited weak glucose repression and the absence of low-affinity glucose transport [69]. This allowed the suggestion that the repression of high-affinity glucose transport is a manifestation of the action of the general cAMP-dependent regulatory system, i.e., the signalling pathway Cdc25–Ras–Cdc35–cAMP–cAMP-dependent protein kinases, and that the K_m of transport proteins is modulated by their reversible phosphorylation [69, 70]. It was also found that the activity of the regulator protein Snf1 is modulated as well and that this process depends on the energetic status of cells (i.e., on the AMP : ATP ratio) and involves proteinase Srf [71].

Another negative regulatory signal is generated by the sensor protein Ggs1, which is identical to one of the subunits of the trehalose-6-phosphate synthase complex encoded by the *GGS1/TPS1* genes. Since Ggs1 protein is bifunctional, it is tempting to suggest that the trehalose-6-phosphate synthase complex, which is activated in the late exponential growth phase, when glucose concentration in the medium decreases, is also involved in the control of glucose transport. In any case, there is experimental evidence that trehalose-6-phosphate controls sugar influx into yeast glycolysis by inhibiting hexokinase [72].

The role of one more glucose-activated regulatory circuit, which includes the signalling pathway Cdc25–Ras1–adenylate cyclase–cAMP-dependent protein kinases, lies in the modulation of the activity of some enzymes through their phosphorylation [73]. One of such enzymes is the isozyme fructose-6-phosphate hexokinase II (Pfk26), whose product, fructose-1,6-diphosphate, is a secondary messenger. Along with AMP, fructose-1,6-diphosphate is the major activator of phosphofructokinase under an excess of glucose. The signal is involved, via Ras–Cdc35, in the activation of the transcription of the *PFK26* and *PFK27* genes [74, 75].

The mechanism of the interaction of glucose with the Cdc25 protein is unknown, although Gross *et al.* showed that the hyperphosphorylation of the Cdc25 phosphoprotein in response to glucose results in its dissociation from Ras1 and in the binding of the latter to GTP [76]. The GTP–Ras1 complex activates adenylate cyclase and thereby cAMP synthesis. Due to the GTPase activity of Ras1, GTP–Ras1 transforms into the inactive complex GDP–Ras1. Therefore, the amount of the active GTP–Ras1 complex is controlled by the proportion between GTPase activity stimulated by Gap protein and the rate of Cdc25 hyperphosphorylation stimulated by glucose [76, 77].

In eukaryotic cells, glucose also induces the turnover of phosphoinositides, which are involved in signal transduction to protein kinases via pathways either dependent on the Cdc25–Ras complex [78] or not [79].

One more regulatory circuit of yeast glycolysis is associated with hexokinases. The protein kinase activity of hexokinase II can initiate a phosphorylating cascade and thus modulate the activity of enzymes responsible for the pleiotropic effect of glucose [80]. It should be noted that the expression of the *HXK1* and *GLK1* genes encoding two other hexokinases is repressed by glucose and cAMP [81].

Glucose also suppresses synthesis of some enzymes involved in the catabolism of alternative carbon sources, oxidative metabolism, and gluconeogenesis. This regulatory system, which operates at the transcriptional level with the pleiotropic regulator protein Mig1, is very sensitive to glucose, is independent of the adenylate cyclase signalling pathway, and likely involves glucose-6-phosphate as a secondary messenger [75].

Bacterial system of universal response to stresses and carbon starvation. It was shown that bacterial cells can respond to various stresses (carbon starvation, transition to the stationary phase, overproduction of acidic metabolites, and to some other stresses by switching on the universal stress response system, which is regulated by the alternative sigma-subunit of RNA polymerase, RpoS (σS or σ^{38}) [82].

RpoS controls about 50 regulons. Thirty of them have been identified, including proteins controlling the cell cycle, synthesis and consumption of reserve substances (glycogen, trehalose, and polyphosphate), defense against heat, osmotic, oxidative and other stresses, transition to anaerobic metabolism, etc. [82]. Together with other universal stress response regulators, such as UspA [83], RpoS-dependent operons are responsible for the stationary-phase bacterial phenotype, including the reduction of ATP content and growth rate and the activation of synthesis of reserve polymers and antistress mechanisms. In *E. coli*, the sta-

tionary-phase phenotype involves the excretion of acetate to the medium. All these events hinder the oversynthesis of pertinent products, which explains the considerable increase in the production of recombinant proteins in the rpoS (katF) mutants of *E. coli* [84]. Using a mutant deficient in the ribosome modulation factor (Rmf), Chou *et al.* have also demonstrated that the production of recombinant proteins is limited by the accumulation of inactive dimeric 100S ribosomes in the stationary phase [84].

Regulation by guanosine tetraphosphate. Efficient producers of amino acids and proteins can be created by blocking the *relA*-dependent regulation and thereby redistributing the metabolic fluxes under conditions of depletion of all or certain of the amino acids from their intracellular pool [85]. In *E. coli*, this leads to the generation of uncharged tRNA, whose interaction with the A site of ribosomes activates RelA, the ribosome-associated guanosine tetraphosphate (ppGpp) synthetase. Guanosine tetraphosphate acts as a secondary messenger activating the expression of all amino acid encoding operons at the expense of the degradation of some cell constituents, such as the protein-synthesizing apparatus, cell wall, and reserve polymers, whose necessity for cells decreases as the growth rate slows down [85]. Actually, this is equivalent to the stationary-phase phenotype, which is unfavorable for protein oversynthesis. It remains unclear how these processes are related to the second ppGpp synthetase, SpoT, which is independent of RelA and ribosomes and is activated by intermediates of the plasma membrane lipids in the stationary growth phase and under conditions of carbon deficiency [86, 87].

Regulation by Ntr-system. The effect of the Ntr-system on syntheses in recombinant strains was considered by Liao *et al.* [35]. In *E. coli* and some other bacteria, the Ntr-regulon controls the assimilation of ammonium present in the medium at low concentrations, when glutamate dehydrogenase, whose K_m with respect to ammonium is too high, does not function [88]. The main regulatory element of the Ntr-system is NtrC protein, a transcriptional regulator of the constituent *glnALG* operon. By forming a binary sensor–regulator system with NtrB protein kinase, NtrC activates the transcription of the genes of glutamine synthetase (*glnA*), protein kinase (*glnL*), and its own gene *glnG*. In this system, in contrast to some other binary regulatory systems, NtrB protein kinase is not the primary sensor, and ammonium is not the primary signal. Actually, the primary signal is an increase in the 2-ketoglutarate-to-glutamine intracellular concentration ratio, and the primary sensor is uridylyl transferase, which is activated by the high 2-ketoglutarate-to-glutamine concentration ratio and uridylizes the next component of the signalling pathway, P_{II} protein, to form P_{II}-UMP. The latter activates the NtrB protein kinase, which, in turn, phosphorylates the transcriptional regulator NtrC. Phosphorylated NtrC initiates the transcription of the *glnALG* operon from the major *glnA* gene promoter. The transcription is mediated by the alternative subunit of RNA polymerase, RpoN, or σ^{54}.

The regulatory control of the Ntr-system over the recombinant phenotype was analyzed by Liao *et al.* [35]. It was shown that, with the genetically enhanced activity of phosphoenolpyruvate carboxykinase (Pck), glutamine synthetase (GlnA) fails to be induced by ammonium deficiency in the medium. The authors believe that the enhanced production of pyruvate initiates the synthesis of an intracellular signal metabolite that coordinates carbon and nitrogen metabolism.

The role of other bacterial regulatory systems. Undoubtedly, in facultative anaerobes, glycolysis is regulated not only by the mechanisms responsible for the Pasteur and Crabtree effects, but also by the Fnr and Arc regulatory systems [89, 90]. As has been shown recently, the transcriptional regulator Fnr is also involved in metabolic control in recombinant microorganisms [91].

In many microaerophilic bacteria, the phosphotransferase system (FTS) of sugar transport, besides being involved in the catabolite repression of the operons controlling the consumption of severely metabolizable carbon sources, plays a significant role in the regulation of glycolysis. The primary signal may be the PEP-to-pyruvate concentration ratio. Therefore, any genetic engineering manipulations that increase the PEP pool may enhance the input glycolytic flux and thereby increase the production of metabolites from PEP within the limits imposed by the phosphotransferase system [35, 92]. The *E. coli* strain in which the PTS of glucose transport has been replaced by the independent PTS of galactose and proton symport capable of nonspecific glucose transfer exhibited a noticeable increase in the glycolytic flux [93].

Of much interest is the regulatory role of molecular chaperons in the production of native proteins in recombinant cells [94, 95], but this topic is beyond the scope of the present review.

CONCLUSION

The foregoing suggests that the theory of metabolic flux control developed by Heinrich, Rapoport, and their followers, which identifies rate-limiting steps by analyzing the kinetic properties of enzymes and the stoichiometry of enzymatic reactions, is appropriate only to peripheral metabolism, as less rigid than intermediary or central metabolism. Furthermore, it is desirable that any bypasses of the major metabolic flux and regulatory feedback circuits be absent. In central and intermediary metabolism, rate-limiting enzymes are usually subject to multilevel regulation (this is especially typical of eukaryotes), which makes the application of the TMFC rather inefficient.

The identification of rate-limiting steps in central and intermediary metabolism should be followed by the rearrangement of the regulatory network in order to

make the identified bottlenecks less rigid. However, analysis of metabolic regulation in *S. cerevisiae* shows that the gain from such rearrangement is doubtful. For example, the enhanced expression of phosphofructokinase in *S. cerevisiae* did not affect the anaerobic glycolytic flux but increased the aerobic glycolytic flux to the level typical of anaerobic conditions [96]. In other words, the Pasteur effect was prevented, but the glycolytic flux did not reach the level typical of the wild-type strain.

There are two possible approaches for elucidating regulatory influences on a rate-limiting step. The first approach is implemented in so-called inverse genetic engineering [97]. In terms of this approach, a rate-limiting step is somewhat modified by genetic engineering, and then the metabolic and regulatory consequences of this modification are thoroughly analyzed to form a basis for the next genetic engineering operation. The desired result (a more efficient recombinant strain) is achieved via a series of successive metabolic modifications. The second approach involves the numerical simulation of a metabolic block with allowance made for its multiple regulation [98]. It is obvious that a combination of these approaches may appear especially fruitful.

The genetic engineering of molecular complexes and cell organelles (replisome, proteasome, and ribosome) is a very important problem [13]. In this connection, we should mention the publication of Carrier and Keasling, who showed the possibility of controlling messenger RNA stability in bacteria [99].

As is evident from the above discussion, the intermediary metabolism of *S. cerevisiae* growing on carbohydrates is very rigid. At the same time, yeasts growing on methanol (see, for instance, [100]), ethanol, aliphatic compounds, and other unusual substrates may appear to be more convenient objects for genetic engineers.

To conclude, further advances in metabolic engineering can be expected in the case of the coordinated development of genetic engineering, classical and evolutionary genetics, and cell biology centered on the investigation of the metabolic organization of living cells and global systems of metabolic regulation.

ACKNOWLEDGMENTS

This work was supported in part by the Russian Foundation for Basic Research grant no. 99-04-04002 and the NWO-RU grant no. 047-007-021.

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