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Microbial cellular biology and current problems of metabolic engineering

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Abstract

Current problems of metabolic engineering and its further successful development is strongly dependent on the profound knowledge of regulatory sphere of host cells and its influence on the expression of foreign genetic information. This is especially important for intermediary and central metabolism of a host cell, which is a key part of the cell metabolism, supplying the target biosynthetic and catabolic processes by metabolites and by the energy. However, intermediary and central metabolism is usually characterized by the most intricate and elaborate regulatory systems, including pleiotropic 'global' sensor and control mechanisms. This regulatory complexity makes this part of a cell metabolic network especially rigid for foreign intervention. This interaction of a host regulatory sphere and recombinant biosynthetic processes is the core problem of the modern metabolic engineering, interfering and restricting its further development.

Keywords: Metabolic engineering; Cell physiology; Metabolic limitation; Flux analysis; Network rigidity

1. Introduction

Metabolic engineering is a result of the synthesis of classical microbial genetics and selection, cell biology and genetic engineering. Advent of metabolic engineering was declared by Bailey in 1991 [1]. His definition of this newborn science was as follows: ''Metabolic engineering is the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory function of the cell with the use of recombinant technology. The opportunity to introduce heterologous genes and regulatory elements distinguishes metabolic engineering from traditional genetic approaches to improve the strain''.

So, the Bailey's definition emphasizes the use of recombinant technology for cell processes manipulation, and confines the scope of metabolic engineering by applied tasks. One can simply see that this creates the barrier between classical genetic selection and metabolic engineering and leaves out of the latter basic research in biochemistry and in cell biology. It was perhaps the contradictions in this definition that stimulated the appearance of some alterna-

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tive terms: ‘metabolic pathway engineering’, ‘in vitro evolution’, ‘directed evolution’, ‘cellular engineering’ and so on [2].

Accepting the term ‘metabolic engineering’ as adequately reflecting the essence of the phenomenon, one should, however, mention that a method used by any science hardly determines the matter of the research. The peculiarity and the main specific trait of metabolic engineering is evidently engineering approach to the research, reconstruction and design of cell metabolic network. Hence, the classic works on genetic selection of microorganisms — producers of different metabolites, if they were carried out not by random mutagenesis, but through precisely targeted alterations in metabolic pathways and regulatory networks, should be considered as predecessors and characteristic examples of metabolic engineering. Besides this, engineering of macromolecules should be regarded in the scope of metabolic engineering and, more than that, some physiological approaches can be included into this field of research. Macromolecular engineering is a separate problem; as to the physiological approaches to metabolic engineering, some examples will be given later on.

Having all this in mind, we consider as a more adequate definition of metabolic engineering that by Nerem [3]: ‘‘Application of the principles and methods of engineering to problems in cell and molecular biology of both a basic and applied nature’’. The author has used for this field of research the term ‘cellular engineering’; however, ‘molecular engineering’ fits to the definition completely, but more widely used nowadays.

It is worth emphasizing that engineering approach can be used to solve applied tasks as well as for basic investigations in biochemistry and cell physiology, where ultimate decisions on the modes of metabolic processes and their regulation can be done only by using the methodology of metabolic engineering [4].

Problems of metabolic engineering are widely discussed to date and are the subject of some comprehensive reviews [2,5–10]. These reviews contain numerous and spectacular examples of purposeful and effective application of metabolic engineering for improved production of chemicals already produced by the host organism, extended substrate range for growth and product formation, addition of new catabolic activities for degradation of toxic chemicals, production of chemicals new to the host organism, modification of cell properties, etc. However, an analysis of these reviews and of a multitude of experimental works suggests the existence of evident misbalance between the capacities of metabolic engineering methodology and the basic knowledge of microbial cell metabolism and, in particular, of its regulation. It is no doubt that the basic knowledge is now and will be in the nearest future the main factor, limiting the further developments in metabolic engineering.

Quite a new problem, realized to date by biotechnologists, is a need for engineering in the intermediary and central metabolic networks to control and to govern the metabolic fluxes in this sphere from the very entry of substrates into the cell. This task is of special complexity, because the multicontour and multilayer regulation is especially characteristic for this part of metabolic system; this problem is reflected in some of the reviews cited above [6–10].

Taking into consideration the limitation of further development of metabolic engineering by the scarcity of basic knowledge in cellular physiology, we concentrate in this review on these problems, with special attention to the aspects of metabolic limitation of cellular fluxes, rigidity of metabolic networks, and the role of global regulatory systems, which influence and ultimately determine the level of recombinant phenotype expression.

2. Metabolic limitation of cellular processes

In the most general sense, the concept of metabolic limitation of cellular processes means

nothing more than a belief of every biochemist in the existence of intracellular mechanisms of restriction of fluxes rates in the cell network. So, one can speak about flux limitation through a simple enzyme consequence, as well as through catabolic or biosynthetic pathways, metabolic blocks, systems and cellular network in a hole. This restricting (limiting) mechanism is believed to be identified, understood, eliminated (or repaired), which may result in the desired elevation of necessary function [11].

Another belief, which is not self-evident as well, consists in the confidence that cellular processes of a different scale, from a flux through a pathway through the general level of cellular metabolism and even macroscopic parameters (culture growth rate, yields of products and so on) are (or may be) restricted (limited) by a more local element, step or site (or, at least, by a few of them) — by certain site(s) of metabolic limitation. Indeed, real scientific experience has shown that the role of metabolic limitation may be played not only by some particular single enzyme, which possesses the lowest level of activity, but is also often carried out by the elements, blocks, metabolic mechanisms or systems of different scale — in a broad range from the particular enzyme through a complex metabolic system [11].

Since Blackman [12] proposed the concept of the 'rate-limiting step' in 1905, the idea of 'limiting steps', or 'bottlenecks' in metabolic pathways, which are also known as 'rate-limiting', or 'pace-maker' steps and 'metabolic limitation sites' is dominating the approach to understand the control of metabolic pathways [11,13]. The basic idea was supported also by the classical study by Monod [14]. In general terms, metabolic limitation is a restriction of metabolic flux through a particular enzyme consequence, metabolic block and/or through cellular metabolic system in general, by the maximal activity of a particular enzyme, set of enzymes, flux rate through a certain branching point (a nod) in metabolic pathways or by the maximal rate of macromolecular interaction [11].

Nowadays, the literature dedicated to the subject is rather voluminous; here are listed only the references to the key and most characteristic reviews and experimental papers [15–19].

It should be stressed that the location of a 'metabolic bottleneck' is not always stable and depends on culture conditions and, specifically, on growth rate [20]. Location of a particular site of metabolic limitation may also depend on conditions, that's why it can 'slide' along the metabolic chain by the alterations of substrates and other conditions [19]. At a steady state, the metabolic flux limiting function can be distributed between the members of enzymatic chain [21,22].

Attempts were made to classify different types of metabolic limitation according to the type of organization of the limiting site. Some authors distinguish 'classical enzymatic limitation' (at the level of a single enzyme or a particular block of enzymes in the pathway), 'physiological limitation' (at the level of more complex metabolic blocks or a physiological systems, such as substrates uptake systems, respiration machinery, energetic metabolism or status), 'molecular biological limitation' (at the level of macromolecular complexes such as protein synthesizing machinery, DNA replisome and so on) [11,23]. In the latter cases, it often remains unclear whether the distribution of the limiting function among several constituents has taken place, or whether the metabolic complex or block is functioning as a single restricting unit [22], or, in a given limiting complex, local limiting step(s) could be identified.

As to the latter case, there were some attempts carried out to identify such a local bottleneck(s) in a metabolic block. So, investigation of a mathematical model of the respiratory metabolism in *Saccharomyces cerevisiae* has suggested tricarboxylic acid intermediates transfer into mitochondrion as a possible limiting step of the overall energy metabolism [24].

Another attempt of this type was carried out to clarify the hypotheses of 'molecular biological limitation', emphasizing the limiting role of

the protein synthesizing machinery level in different protein synthesis, including an array of recombinant ones [23]. Some authors insisted that the actual limiting factor in protein synthesizing machinery was a number of free ribosomes [25]. It is necessary to have in mind, however, other possibilities: so, in mammalian cells relation of [ADP]: [ATP] and [GDP:GTP] concentrations probably limited a protein synthesis at the translational level [26].

Successes in design of specialized ribosomes for the synthesis of some particular polypeptides have shown to date that interactions of specific macromolecules could be the processes restricting the overall efficiency of protein synthesis. The efficiency of these processes could be emended: for instance, interaction of mRNA and ribosome can be activated by introducing pertinent mutations into the 'anti-Shine-Dalgarno' region of 16S rRNA. The translation initiation level could be also increased through design of hybrid protein genes and hybrid operons [27,28].

However, one should also bear in mind that the constituents of tentative limiting molecular complexes and blocks can be so tightly integrated by regulatory bonds and signals that, practically, it seems impossible to identify and distinguish some local limiting metabolic steps or interactions. A limiting block of this kind can be, for instance, a metabolic chain of some extent with a lack of pronounced local limiting sites, i.e. 'the bottle neck' function is almost evenly distributed among its components. Another plausible example of a similar kind, macromolecular complex, is organized by a multiple contours of reverse stabilizing bonds. So, the situation could be similar to that in protein synthesizing machinery, where some proteins and ribonucleic acids are subjected to different modes of autogenous control [29]. In such cases, the task of precise identification of a local site of metabolic limitation and its compensation or modulation by means of genetics technology approaches appears to be a rather sophisticated problem.

3. Identification of the local sites of metabolic limitation — intuitive period

Cited data and considerations duly demonstrate that identification of limiting sites in any case is a difficult task. However, this is, unfortunately, the main prerequisite for effective design for successful procedures of metabolic engineering. It should be mentioned that, especially at the early steps of genetic engineering history, the identification of 'bottle necks' was often carried out intuitively, just on the ground of all the relevant knowledge on the problem and general consideration concerning metabolic routs and regulation. However, accumulated experience cannot be ever considered successful.

The work by genetic engineers from ICI can be regarded as a spectacular manifestation of this sort of methodology: designing a methylotrophic single cell protein producer, the bacterium *Methylophilus methylotrophus*, with a modified system of ammonia assimilation [30]. The idea was to substitute ATP-consuming glutamine synthetase, operating in the bacterium, by ATP-independent glutamate dehydrogenase, taken from another organism, *Escherichia coli*. It was proposed that ATP saving was permitted to increase extra carbon source assimilation and, hence, to enhance the biomass yield.

Frankly speaking, the author's logic was not quite transparent, because $NADP^*H$ consumption by glutamate dehydrogenase and its extremely high K_m for ammonia did not permit to achieve the expected surplus. This was perhaps the reason why the extra biomass yield was not higher than 4%–7%; however, the work has gained wide recognition in scientific literature.

One of the favorite approaches in identifying metabolic 'bottle necks' during this 'intuitive' period was the identification of correlation or kinetic analogy of some macroscopic parameter and certain metabolic function or cell constituent. For instance, glucose uptake by *E. coli* cells, growing in glucose-limited continuous culture, was suggested to be a metabolic step, limiting the culture growth and restricting the

Table 1
Some typical examples of metabolic limitation site identification and location — general ideas

Limited parameter	Limiting site	Organism	References
Culture growth rate	PTS^{gle} activity	*E. coli*	[15,31]
Culture growth rate	PTS^{gle} activity	*Klebsiella aerogenes*	[33]
Culture growth rate	substrate (methanol) uptake rate	*Methylotrophic bacteria*	[34]
Glycolytic flux rate	glucose transporter used	*Trypanosoma brucei*	[35]
Culture growth rate	respiration activity	*E. coli*	[16]
Culture growth rate	respiration activity	*S. cerevisiae*	[17]
Culture growth rate	energetic cell status	*Klebsiella pneumoniae*	[32]
Intracellular lipids synthesis	phosphofructokinase activity	*Rhodosporidium toruloides*	[19]

maximal growth rate [15,31]. The reason for this conclusion was the analogy in kinetic variation of phospho-*enol*-pyruvate dependent phosphotransferase system (PTS) of glucose transport into the cells and of the culture growth rate in a chemostat. However, the discovered regularity appeared to be valid only at the range of low glucose concentrations; in other cases in the cells, besides PTS, some other uptake systems, transporting glucose are operative [32].

It should be stressed that the scope of this review is supposed to cover the limitation processes and sites: firstly, localized in an intermediary metabolism of microbial cells and in the central metabolic pathways, and, secondly, controlling predominantly large metabolic blocks, particular divisions of metabolism, the general level of the cellular metabolic network and macroscopic parameters of vital activity of cell culture. It is well known that from the 1950s to the present, there were lots of spectacular examples of successes achieved by metabolic engineering in the peripheral sphere of cell metabolism, predominantly in the biosyntheses of low molecular weight metabolites and some catabolic processes. It is made possible, especially in bacteria, because of a comparatively simple regulatory system in this sphere. This early work is beyond the scope of this review, and will be concentrating on the problems and perspectives of molecular engineering at the level of intermediary and central metabolism, which are the backgrounds for different peripheral secondary functions.

Some of the mostly characteristic data accumulating in this field of metabolic engineering during the period of predominantly ‘intuitive’ metabolic network analysis and limiting sites identification are given in Table 1. They permit to feel the dominating ideas: the possible limitation at the level of primary substrates entry into the cell [15,31,33–35], the importance of the supply of biosyntheses by energy and the possible limitations by these factors [16,17,36,37], the possible limiting role of particular regulatory enzymes of central metabolic pathways [19], the crucial role of the protein synthesizing machinery level for efficiency of proteins and cell mass production [23,25].

However, the realization of the complexity of the problem was reflected in the paper with a characteristic title: ‘‘Metabolic growth rate control in *Escherichia coli* may be a consequence of subsaturation of the macromolecular biosynthetic apparatus with substrates and catalytic components’’ [38].

4. Metabolic limitation research after Kacser and Burns [39]

Presently, several different approaches to the analysis of intracellular metabolic fluxes and the identification of metabolic ‘bottle necks’ have been developed and are still being developed. These approaches are substantially different not only methodologically but conceptually as well. There is no urgent need to consider all of them

in great detail, so we shall pay particular attention to the most popular ones.

The especially great expectations were connected with the use of the so-called Metabolic Flux Control Theory (MFCT, or FCT), whose development was connected with the well-known works by Kacser and Burns [39], Heinrich and Rapoport [21] and, a little later, by Fell et al. [13,40,41].

The central idea of the FCT is based on the axiom that enzymes of the metabolic routes are saturated by the substrates differently and, consequently, possess different capacities to increase the metabolic flux through the pathway. So, they have to play different roles in the flux control. Knowing the catalytic characteristics of every enzyme of the pathway, as well as intracellular metabolites concentrations and their possible regulatory influences, one can estimate the contribution of every enzyme into the general control of the flux through the pathway.

Metabolic control analysis defines the quantitative link between the flux through a pathway and the activity of an enzyme in terms of the flux control coefficient. Roughly, the flux control coefficient represents the percentage change in flux divided by the percentage change in the activity of an enzyme that was responsible for the flux change [41]. Typically, the relationship between a flux and the amount of enzyme is approximately hyperbolic, although this cannot be formally guaranteed. At very low enzyme levels, on the near-linear part of the hyperbola, flux increases almost proportionally with the amount of enzyme, and the flux control coefficient is close to 1. However, the flux control coefficient progressively decreases as the flux-enzyme hyperbola flattens out and approaches 0 near the plateau.

If the flux control coefficient were close to 1, the enzyme could be classed as a ‘rate-limiting’ or ‘pacemaker’, but the theory shows that such values are unusual. The so-called summation theorem [39] states that if the flux control coefficient of all the enzymes in a metabolic system on the particular flux is added up, the sum is 1.

The flux summation theorem also highlights the fact that the flux control coefficient of an enzyme is not an intrinsic property of that enzyme alone, but a system property. If flux control coefficient of an enzyme E changes, as its activity is increased from very low level to very high ones, it is apparent that if this flux control coefficient is changing, so must be the coefficients of enzymes whose activities have not been changed, in order that the summation total for the flux control coefficients remains at 1 at all levels of E (this section was cited from Ref. [41]). According to Fell [41], ‘‘a major reason for the relative lack of success in increasing metabolic flux by over-expressing a single enzyme (in the absence of pleiotropic effects on the activities of other enzymes) has been the reliance on traditional biochemical dogma about the existence and identity of rate-limiting steps. Metabolic control analysis has shown that the control of flux is likely to be distributed over a number of enzymes, with any particular enzyme having a limited influence in most circumstances’’.

Putting it all together, one has to conclude that the orthodox Flux Control Theory had led the problem of metabolic engineering aside from the concept of local metabolic limitation, which was expected to be a promising prerequisite to regulate intracellular metabolic fluxes for practical uses. It is not surprising that Bailey et al. [42] in 1996 proclaimed unequivocally: ‘‘The classical method of metabolic engineering, identifying a rate-determining step in a pathway and alleviating the bottleneck by enzyme overexpression, has motivated much research but has enjoyed only limited practical success. Intervention of other limiting steps, of counterbalancing regulation, and of unknown coupled pathways often confounds this direct approach’’.

Actually, the pessimistic statements of the supporters of orthodox FCT, sounded more often recently, do not adequately reflect the real situation in the problem. It should be reminded that from the very beginning there were a lot of criticism of the FCT principal starting points,

pretending to be able to describe the real network dynamic of metabolic intracellular fluxes, inferring this from kinetic characteristics of the particular enzymes as they are in vitro. Many opponents have pointed out as early as in the late 1980s that the theory was developed, actually, only for unbranched metabolic pathways, it had paid too little attention to regulatory connections and forces, operating in a particular metabolic block, which, in many cases, can be multicontour and multilayered [11]. ''Flux control coefficients have not included the effect of the regulators that interacted with the control enzymes. Consequently, these coefficients do not provide sufficient information for comparing the relative importance of control sites. It is not possible to deduce the relative importance of control mechanisms or control sites under physiological conditions simply by comparing the magnitudes of these control coefficients'' (Crabtree and Newsholme, [43]). The most important consideration was that by Masters [44], who has stressed an inadequacy Michaelis kinetics, developed for in vitro systems and for enzymatic reactions in solutions, applied for highly structured and compartmentalized even prokaryotic cell plasma. These are, perhaps, only a fraction of the reasons why many cases of 'an unsuccessful prediction from pathway analysis' were openly declared by some investigators [45].

A distinctive alternative concept of 'metabolic flux analysis' was suggested by Crabtree and Newsholme [43]. Their central point consists of the statement that the experimenter, having in mind the identification of the limiting steps of cellular fluxes, must just measure the other things in the cell, not those investigated by the proponents of the orthodox FCT. ''An alternative approach is to measure the overall response of a flux and its associated metabolite concentrations to a stimulus, and then compare this with the same response calculated theoretically'' [43].

A convincing and practically quite acceptable example of a similar approach to flux analysis and control of the central metabolic pathways in *E. coli* was demonstrated by Holms [46]. The methodology, as the author has it presented, was simple: ''When you measure utilisation of carbon source, production of biomass and other products and growth rate then, provided you also know the metabolic routes which operate, you have all the data required to construct a quantitative description of all the reactions (net) which sustain these processes. Such a description contains the flux through every enzyme in the central metabolic pathways''. Growing the culture in a steady-state conditions on different carbon sources, and being able to measure substrates and products of all the participating reactions and to calculate the rate and efficiency of each elemental and all the net conversions of substrates into products, including intermediates, you actually are estimating the rates of fluxes through every metabolic step. Laying the data obtained on the metabolic map, it is easy to localize most of the slow steps [46].

Currently, the major analytical tool for performing real-time analysis of metabolic flux and energy metabolism is nuclear magnetic resonance spectrometry (NMR) [7]. One drawback of in vivo NMR analysis is that high cell densities are required to obtain the necessary sensitivity, but new improvements, including specially designed reactors and the use of biosynthetic fractional ^{13}C-labeling of amino acids coupled with two-dimensional NMR, are opening new possibilities and some important practical results were already achieved (reviewed in Refs. [7,47].).

Besides the ones mentioned above, now there are several similar and/or intermediary variants of the theory of control and analysis of cellular metabolic fluxes [8,45,48–50]. Simultaneously, the application of mathematical tools is becoming all the more and more conventional and sophisticated [24,40,48]. There have appeared a lot of useful empirical experimental procedures, permitting tentatively to locate plausible sites of metabolic limitation, such as: dynamic control of intracellular intermediate pools during a cel-

lular response of the growing in a batch condition culture to the sudden addition of some different substrates [51]; measurement of metabolite concentrations in a transient states of culture growth with a deducing possible limiting enzyme [52]; particular enzymes titration by specific inhibitors [53]; comparison of growth kinetics of the culture, growing on mixtures of substrates, including some selected metabolites [54]; extracellular analysis of metabolites in a culture medium in different growth conditions [55]; and many compiled approaches, e.g., Refs. [9,56].

Summarizing the results accumulating up to date, there were evident successes and those not always understood fail, one can cite the title of a paper: ''All models are wrong, but some are useful'' [57].

5. 'Bottlenecks' compensation: different approaches and some preliminary considerations

Methods of classical genetics and selection have resulted in successful decisions of many of the most important applied tasks and fundamental problems of metabolic engineering: to direct intracellular metabolic fluxes to the enzymes, responsible for the synthesis of target products; to cut off and/or minimize the side branches of fluxes, wasting the intermediates; to switch off most part of the negative regulatory contours; to prevent conversion of the target products into other metabolites. All the history of the industrial amino acids production is a spectacular example of this type of work.

However, classical genetic methodology permits only to reach maximal (theoretical) activity and productivity of metabolic cell system of a cell, conditioned and encoded by its genotype. The capacity of the cell to extend this level is limited.

Genetic and protein engineering is universal and is the most powerful tool to achieve such level, which can be counted many times compared to the original. It is not always, however, realized that some problem of this sort can be solved by some rather simple physiological methods.

So, using simple cyclic algorithm of continuous cultivation of strains-producer of some proteins, with periodic changes of the growth culture rate from minimal to the maximal one, appeared to be possible to very effectively exploit the protein synthesizing machinery of the bacterial cell for a particular protein production (including the recombinant one), avoiding inevitable enormous maintenance carbon and energy expenditures, characteristic of both chemostat and batch culture [58].

Another typical example — properly designed process of co-metabolism. Traditional process in this field — production of different acids by biotransformation (partial enzymatic conversion) cyclic hydrocarbons, such as benzene, xylenes, pyridines, polycyclic compounds and their derivatives [59]. The traditional 'bottlenecks' in this reactions — limitation of monooxygenases of primary attack (hydroxylases) activity by a shortage of reduced nucleotides. It was shown that carefully selected co-substrates, which are simultaneously oxidized by the same culture and generates necessary cofactors, highly stimulated corresponding oxygenase and enhanced products yields [60]. In the most spectacular case, oxidation of 3-methylpyridine to nicotinic acid by the non-growing cells *Nocardia minima* significantly accelerated, when the culture was oxidizing in parallel some pentoses (xylose, arabinose) to corresponding acids, supplying the primary oxidation of methyl substituents by reducing equivalents, but without further degradation of arising xylonic or arabonic acids [61].

To balance the assimilation processes of carbon and energy in growing yeasts, Babel et al. [62], using the mixtures of carbon-rich but energy-excess carbon sources (methane, methanol, ethanol, *n*-alkanes) and energy-deficient substrates (glucose, acetate, formate), have attained

the maximum carbon conversion efficiency in different bacteria and yeasts.

A classical example of physiological methodology application for solving metabolic engineering tasks is the generation of bacterial strains-superproducers of some enzymes by the prolong selection in chemostat. The first outstanding work of this type has been done as early as 1961 [63]. The authors have obtained *E. coli* strain — superproducer of β-galactosidase. This work has actually opened a new trend in modern biotechnology and metabolic engineering — evolutionary biotechnology.

However, having in mind all the somewhat attractive possibilities of physiological and evolutionary approaches in metabolic engineering, one has to admit without any doubt that the central, most popular and most powerful tool of this technology is the use of genetic engineering methods on the basis of precise knowledge of cell physiology, metabolic network function and dynamics of intracellular metabolites fluxes, revealing the targets for the application of genetic engineering methodology.

6. Problem of network rigidity

With the advent of metabolic engineering, biotechnologists have discovered for themselves that microorganisms used as the hosts in recombinant design differ also by their metabolic rigidity [64]. Different metabolic blocks and/or systems of the same organism can also have different levels of rigidity. Stephanopoulos and Vallino [64] presented a rather general definition of the metabolic, or cell network rigidity as an 'inherent resistance to flux alterations'. Specifically, they have emphasized the conservative type of fluxes distribution in branching points, so-called nodes and deviations, where regulatory mechanisms strictly control the relation of fluxes rates in ramifications.

It is easy to see that besides the strict control of fluxes in nodes, a variety of other regulatory phenomena can be responsible for the metabolic rigidity: general maximal enzyme activities in the consequence and the absence of the pronounced sites of metabolic limitation; operation of many negative regulatory contours in a pathway; function of complex, pleiotropic, 'global' regulatory systems, which resulted in the behaviour of complex metabolic blocks and systems as a single regulatory units; and so on.

A good example of rigidity difference between separate metabolic blocks of the same organism gives a comparison of glycolysis and tricarboxylic acids cycle (TCA) systems in facultative anaerobes, such as *E. coli* and *S. cerevisiae*. So, Lee et al. [65], to determine the role of the tricarboxylic acid cycle in *E. coli*, have investigated the flux distribution in a mutant *gltA*, lacking citrate synthase activity. The authors have found that even though carbon flux from glycolysis was unable to enter the TCA cycle, the amount of ATP produced in glycolysis was comparable to that generated in the wild-type cells. The paradoxical conclusion was that the TCA cycle in *E. coli* plays a primarily biosynthetic role, and ATP is produced from glucose mainly through glycolysis and acetate pathways in aerobic condition as well as in anaerobiosis.

It should be noted that these data could be interpreted in quite another way as well. The mutant with blocked TCA cycle actually is identical physiologically to the wild type in an anaerobic condition, where metabolic flux through glycolysis is increased several times under the action of global regulatory system, well known as the 'Pasteur effect'. This is the way to compensate the energy deficiency, which resulted from an impaired Krebs cycle. Very likely, the same picture can be observed also in aerobiosis with high glucose concentration ('Crabtree effect').

The data presented have shown evidently a high plasticity of glycolytic system and the broad range of glycolytic flux modulation, which is a prerequisite for cell energy apparatus adaptation in a condition of changeable oxygen concentration. Activation of glycolytic ATP genera-

tion, demanding increasing glycolytic flux, results in the efflux of intermediates into the medium, because they surpass the metabolic capacity of a more rigid and strictly regulated TCA cycle [66].

7. 'Bottlenecks' compensation in intermediary and central metabolism of facultative anaerobes

All these peculiarities, characteristic for the life strategy of facultative anaerobes, open the broadest ability to manipulate by general level of glycolytic flux, not wasting its surplus in futile energetic cycles, but redirect certain fluxes into the branches, leading to many metabolites of great practical value. That's why the list of examples of successful compensation of 'bottlenecks', found in a main pathway as well as in ramified lateral enzymatic chains, which are leading to a significant increase of products yields, is rather numerous in metabolic systems, controlled by the regulatory mechanisms like 'Pasteur effect' among facultative anaerobes.

As to the central metabolic mechanisms (such as TCA in these microorganisms), maintaining of their homeostasis is, evidently, the most important task for every organism. This is confirmed, by the way, by the communication, that membrane ATP-synthase gene deletion in *E. coli* has resulted in only a slight decrease in growth rate and biomass yield of the culture [67]. In aerobic conditions, this results in TCA tough rigidity as well. This is, evidently, the reason, why only a few successful works of TCA manipulation are known. So, Liao et al. [68], systematically studying the TCA flux regulation, have found that the over-expression of these enzyme caused metabolic imbalance and severe disturbance to global regulation in *E. coli.* Overproduction of fumarate reductase in *E. coli* resulted in improved conversion of fumarate to succinate, accumulated into the medium [69], or in induction of a novel intracellular lipid-protein organelle, without any effect on the general metabolism and/or the growth parameters of the culture [70]. It is interesting that overexpression of some enzymes, producing TCA intermediates, e.g., phosphoenolpyruvate carboxylase, significantly (3–5-fold) increased the yield of succinate, evidently, because this conversion is on the gluconeogenic pathway. In contrast, overexpression of phospho-*enol*-pyruvate (PEP) carboxykinase had no effect [71]. A similar picture was observed in yeast as well: overexpression of cytosolic malate dehydrogenase (MDH 2) caused a 6–16-fold increase in cytosolic MDH activity, but only 3–7-fold increase in malic acid accumulation in the production medium and also caused an elevation in the accumulation of fumaric and citric acids, which did not have a noticeable influence on energetic metabolism or growth [72].

Contrary to the central metabolism, intermediate metabolic pathways in facultative anaerobes, particularly glycolysis and associated pathways, leading to many practically important metabolites, are much more flexible and accessible to targeted engineering manipulations. It is well known that one of the important points of flux regulation is the junction between glycolysis and the TCA cycle, which includes enzymes interconverting PEP, pyruvate, and oxaloacetate. These enzymes are important for directing metabolic fluxes to various lateral biosynthetic pathways. A lot of practically useful works have been done this way, using amplification of homologous as well as cloned active heterologous genes and concentrating primarily on the production of alcohols, amino acids and different products of fermentation, including substances, previously not produced by a particular organism and/or utilized as substrate. So, *E. coli* PEP-carboxylase gene amplification in *Serratia marcescens* strain, already bearing several regulatory mutations, and similar operation in *Brevibacterium lactofermentum* have resulted in a remarkable increase of threonine biosynthesis (up to 63 g/l) [73,74]. Some of the most characteristic examples of this type of processes are

Table 2
Some examples of metabolic flux modulation in facultative anaerobes

Target	Method used	Organism	References
Threonine hyperproduction	PEP-carboxylase gene amplification	*Serratia marcescens*	[73]
Threonine hyperproduction	PEP-carboxylase gene amplification	*Brevibacterium lactofermentum*	[74]
Ethanol production from xylose	cloning of pyruvate decarboxylase gene from Zymomonas mobilis	*Klebsiella planticola pyruvate-formate lyase* mutant	[75]
Increased butanol production	activation of butyraldehyde dehydrogenase gene expression	*Clostridium aceto-butylicum*	[76]
Increased ethanol yield	cloning of pyruvate decarboxylase gene	*E. coli*	[77]
Ethanol production from glucose, xylose and lactose	cloning of pyruvate dehydrogenese and alcohol dehydrogenese genes from Zymomonas mobilis	*E. coli*	[78]

presented in Table 2 [73–78]; exhaustive information can be found in recent reviews [2,6,7].

8. Role of global regulatory systems

According to conventional metabolic engineering methodology [45], ''Pathway engineering for production of biochemicals usually starts from terminal steps leading to the desired product. However, the production rate and yield are ultimately limited by the ability to channel carbon flux from the central metabolism to the biosynthesis pathways. After bottlenecks in the terminal pathways are removed, central metabolism, which supplies precursors and energy for biosynthesis, becomes limiting. Because of its critical roles in cellular physiology, central metabolism is highly regulated and its alteration may be resisted by the cell or cause severe perturbation in cellular functions'' [45].

It is quite clear from above that the compensation of a single particular limiting site, or a set of them in a metabolic pathway of central and intermediary metabolism, even removing evident negative regulatory circuits, is not usually sufficient to speed up the flux to the maximal level or near it. That's why the methodology of global control systems management and engineering is recognized now as the next and urgent task in metabolic engineering. Analysis of a variety of these system is too voluminous for the scope of this review; later on some of the most characteristic examples will be presented.

8.1. PTS system in enteric bacteria

PTS system in enteric bacteria is responsible for the uptake of some sugars. As such, it can control the rate of general flux in central metabolic pathways. Hypotheses of this type have been already mentioned [31–33]. But a principal feature of the PTS is the dependence of its activity and, hence, of a sugar flux into the cell, on the supply of this process by the PEP energy, generated in glycolysis. This is the reason for glucose consumption rate stimulation by PEP-generating enzymes (PEP-synthase and PEP-carboxykinase) and for the inhibition of glucose consumption rate by the PEP-consuming enzymes (pyruvate kinase and PEP-carboxylase) [45]. These results can be attributed to the potential control of the PEP/pyruvate ratio on the rate of PTS [45].

The dependence of the glycolytic flux at the level of glucose uptake on the PEP/pyruvate ratio has been avoided by several ways. In a chemostat culture, *E. coli* strains were selected, which transfer glucose by alternative transport systems without the use of PEP [79]. The authors have demonstrated the utility of these

strains for the production of the precursors of aromatic acids. This paper illustrates, by the way, the importance of selection and 'evolutionary' approaches in metabolic engineering [79].

By recycling pyruvate to PEP and directing carbon flux to the production of PEP and erythroso-4-phosphate, the production of aromatic amino acids approached a maximum yield of 86% (mol/mol) from glucose [80]. The same authors [81] have tested xylose, a sugar transported into *E. coli* independently from the PTS, as a substrate for the production of aromatic acids. Because the PTS is not involved with xylose assimilation, the yield of aromatic acids on xylose should be comparable to the yield on glucose, without having to recycle pyruvate back to PEP. Indeed, this hypothesis was verified and a yield of 71% (mol/mol) on xylose was obtained [81].

Comparison of phenylalanine-producing recombinant *E. coli* strain, which has a wild-type PTS, with a mutant utilizing a proton-galactose system for non-specific glucose uptake, has shown that the latter had a higher flux through pyruvate kinase and TCA cycle [82].

These works demonstrate that the PTS is a critical component in the glycolysis, which must be considered attentively when designing metabolic pathways of the relevant bacteria [80].

Besides the PEP/pyruvate stoichiometry, regulating the glycolytic flux in enteric bacteria, catabolite repression and inducer control of many operons is mediated by the PTS. It is well known that the synthesis of cAMP, one of the major regulatory secondary messengers in many microorganisms, in enteric bacteria is controlled by allosteric activation of corresponding enzyme, adenylate cyclase, with the phosphorylated form of the PTS component IIA^{glc}, while the responsive permeases and catabolic enzymes are inhibited by free (non-phosphorylated) IIA^{glc} [83]. One dephosphorylated, the free IIA^{glc} protein inhibits the activity of many enzymes, involved in alternative carbon sources catabolism (lactose, galactose, maltose, raffinose permeases, glycerol kinase, arabinose isomerase). Similar role plays complex cAMP–CRP (CRP for 'cAMP receptor protein').

So, the proper PTS management, using enteric bacteria and some Gram-Positive, possessing this uptake system, is one of the primary operations in metabolic engineering of these bacteria.

Besides these, recent studies have revealed that *E. coli* and *S. typhimurium* utilize a mechanism that is completely independent of the cAMP–CRP complex to control transcriptional initiation of genes, encoding for the key enzymes in the major pathway for carbon metabolism [84]. The pleiotropic transcriptional regulatory protein of this system, Cra ('catabolite repressor/activator'), previously labeled as FruR, influences the utilization of dozens of carbon sources [83,84].

8.2. Carbon source starvation response

Depletion of a carbon source in the cultivation medium, entering of the culture stationary phase, osmotic shock impact or accumulation of the acidic substances, which are often the target products of the fermentation, activates the global system of the cellular response to carbon starvation, or the stationary phase physiological cell response [85]. The universal regulator of this system is an alternative mRNA polymerase subunit RpoS (σS or σ^{38}) [85].

RpoS controls nearly 50 regulons, only about 30 of them are identified up to date. Of the protein products, coded for by these genes, from the point of view of the problem under consideration, of special interest are some proteins of the cell cycle control, of the reserve materials synthesis and utilisation (glycogen, trehalose, polyphosphates); protein, protecting cell against some stresses (heat-shock, osmo- and oxystresses), and also some regulators, switching the cell metabolism on the anaerobic type) [85]. So, the RpoS dependent operons, as well as some other regulators of the so-called universal stress response, especially *uspA* [86] are responsible

for the stationary phase phenotype, which is typical for this state of low growth rate and macromolecular synthesis, low ATP level and production rate, activation of the fluxes to reserve materials accumulation and of stress protection, and (in *E. coli* and some other facultative anaerobes) excretion of acetate and accompanied by low molecular metabolites. Evidently, this physiological state does not fit properly to the fulfillment of the main function of recombinant biosynthesis — hyperproduction of some particular compounds. This was convincingly shown with the use of the *rpoS (katF) E. coli* mutants, which have demonstrated distinct increase in recombinant protein production [87].

The paper cited [87] has shown also that ribosomes inactivation and 100S-dimer accumulation, characteristic for the *E. coli* stationary phase, also restricts recombinant synthesis, but this effect can be repaired by the mutation of *rmf* gene, encoding for the synthesis of a particular protein 'ribosome modulation factor' [87].

8.3. Possible effect of the stringent control and of ppGpp regulation

In some projects, especially by the amino acids hyperproducers construction, there is the evidently urgent need to block *relA*-dependent regulation, which is the mechanism responsible for the redirection of metabolic fluxes and activated by the intracellular amino acids deficiency (see for review Ref. [88]).

Enhanced biosynthesis of some amino acid or protein as target products naturally results in the intracellular amino acid pool depletion or in some particular amino acid deficiency. This brings about uncharged tRNA accumulation. The latter contacts and interacts with the ribosome A-site, activating by this the specific enzyme, RelA-protein. RelA is a guanosine tetraphosphate (ppGpp) synthetase; its product, ppGpp, is one of the highly important secondary messengers into the cell. It functions as the transcriptional activator of amino acids biosynthetic operons; their expression is carried out at the expense of some cell constituents, specifically — of RNAs and proteins — components of the protein-synthesizing machinery, cell envelope and reserve materials, corresponding to the new, decreased growth rate [88]. In fact, this process is analogous to the stationary phase phenotype formation, with all the undesirable consequences for a recombinant synthesis.

One of the important problems remaining to be solved in this general picture of ppGpp regulation — the role and the influence on a recombinant biosynthesis of the second ppGpp synthetase — is SpoT, which is induced independently on RelA. SpoT is, probably, not associated with ribosomes and is induced by some intermediates of cell membrane synthesis in the cytoplasm, maybe, by some fatty acid(s). This process is also associated with stationary phase and, contrary to the synthesis of RelA, activated by carbon source depletion [89,90]. This regulation almost certainly may play a negative part during recombinant synthesis, but the role of SpoT is not yet properly studied.

8.4. NTR regulatory system in engineered bacteria

There are yet a few experimental data on the possible role of this global system in recombinant strains. Nevertheless, its part can be significant. The present data are reviewed in Ref. [45].

Ntr, regulon in *E. coli* and some other bacteria, controls ammonia assimilation when its content into the medium is low. Enzyme glutamine synthetase is responsible for the process, as conventional glutamate dehydrogenase is not operating because of too high K_m for ammonia [91]. The principal regulatory element of this global system, NtrC protein, is a transcriptional regulator of glnALG operon in NTR; it functions as a typical response regulator of two-component systems and interacts with protein

kinase sensor NtrB. However, contrary to the most part of two-component bacterial systems, NtrB protein kinase does not play the role of primary sensor of ammonia concentration and the primary signal generator. The primary signal in this system is the increase of intracellular 2-oxoglutarate/glutamine contents ratio, and the primary sensor — enzyme uridilyl transferase, which is activated by a high 2-oxoglutarate/glutamine ratio and urydilates the next component of the signal-transducing pathway protein P_{II}. The product of this reaction, P_{II}-UMP, activates the protein kinase NtrB, which phosphorylates the response regulator NtrC. Phosphorylated NtrC finds a corresponding enhancer site near the glnALG operon and activates the transcription of these genes, coding for glutamine synthetase, NtrC and NtrB [91]. The transcription is carried out by RNA polymerase with alternative σ subunit RpoN, or σ^{54}.

An example of NTR participation in the control of recombinant genotype expression is shown in Ref. [45]. It was discovered that in recombinant strain with increased expression of PEP carboxykinase, the major enzyme of NTR glutamine synthetase was not induced by ammonia deficiency in the medium. The authors suggested that increased production of pyruvate has resulted in the synthesis of some yet unknown intracellular signalling compound, which coordinates carbon and nitrogen metabolism of the recombinant [45].

9. Discussion

Experimental analysis of the real cellular metabolic flux remains the basic tool of metabolic engineering. However, identification of the limiting cites in the pathways of intermediary and central metabolism provides only the basis for further investigation of the regulatory sphere, which controls the network of these identified and yet unknown 'bottlenecks'. Considering the problem formally, one could conclude that the next stage of the engineering work should be devoted to identification and reconstruction of all functioning regulatory circuits and signals to successive disclose and to bare the stoichiometric 'skeleton' for effective compensation of 'bottlenecks'.

However, the accumulated experience has shown that, in many cases, highly rigid regulatory system, with many parallel contours, circuits and shunts makes this methodology impossible. This is especially important for eukaryotic microorganisms. For instance, in *S. cerevisiae* glucose as a substrate generates a lot of negative back regulatory signals; for our consideration, it will be enough just to list them: (1) interaction of the substrate with the transport proteins results in increasing their K_m for glucose [92–95], (2) glucose initiates the signaling cascade of the proteins CDC25-RAS-CDC35 (adenylate cyclase)-cAMP-dependent protein kinases with many and not always clear positive and negative effects [94–97], (3) yeast hexokinase II possesses protein kinase activity, which suggests a possible function of another regulatory phosphorylating cascade [98], (4) protein glucose sensor Ggs1, being a subunit of trehaloso-6-phosphate synthase, involves intracellular trehalose and trehalose-6-phosphate into flux regulation, inhibiting hexokinase function [99], (5) glucose represses synthesis of some enzymes at the level of transcription through the action of pleiotropic regulator Mig1 [100].

It is no surprise, then, that increased expression of phosphofructokinase in *S. cerevisiae* has not resulted in glycolytic flux rate changes in anaerobiosis, but in aerobic conditions enhanced it to anaerobic level [101]. In other words, the authors removed the Pasteur-effect action, but could not influence the maximal flux. It should be emphasized that in eukaryotes, not only central metabolic pathways but also those in peripheral metabolism often display high rigidity. For example, the authors in Ref. [102] have managed to increase the flux rate through tryptophan biosynthetic system in yeast only after increasing expression of all fife enzymes of the pathway.

In general, analysis of recent experience in metabolic engineering has shown distinctly that classical engineering quantitative analysis and design should be supplemented also by the classic method, known as 'attempts and failures'. Bailey et al. [103] have proposed the methodology of 'inverse metabolic engineering', which suggests a successive genetic engineering of the supposed major limitation site, investigation of metabolic, stoichiometric and regulatory peculiarities of such recombinants in different hosts; then, the next engineering operation on the other site on the basis of new obtained information and so on, until achieving the desired results. As a matter of fact, this approach was applied at the very beginning of metabolic engineering [104]. Another approach, the mathematical modeling of metabolic systems, taking into account the regulatory sphere, can be useful also [105].

The most important problem is engineering at the level of molecular-biological limitation (term by Koplpv and Cooney [23]), i.e., in a sphere of the macromolecular metabolism, biology of complexes like ribosome and all the protein-synthesizing machinery, replisome, proteasomes and so on. Besides the works cited above on specialized ribosomes design [27,28], one should mention the data and general idea of increasing mRNA stability in bacteria [106].

There is no doubt also that the problem of global regulatory systems is more broad and complex than it might seem after a rather short consideration above. There is no doubt that in bacteria FNR and ARC systems, controlling enzymes, responsible for aerobi–anaerobic switches, and especially heat-shock response with developed system of molecular chaperones synthesis, are of great importance in metabolic engineering [46,107–109].

The highest rigidity of glucose consuming yeast metabolism attracts attention to alternative hosts — methylotrophic and ethanol utilizing yeast [110].

In conclusion, one should stress in another time the importance of balanced use and application of genetic engineering, classical genetics and selection, cell biology and culture physiology for successive development of metabolic engineering.

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