Biochimica et Biophysica Acta 1827 (2013) 1039–1047

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Design and analysis of metabolic pathways supporting formatotrophic growth for electricity-dependent cultivation of microbes☆

Arren Bar-Even *, Elad Noor, Avi Flamholz, Ron Milo **

Department of Plant Sciences, The Weizmann Institute of Science, Rehovot 76100 Israel

ARTICLE INFO

Article history:
Received 6 August 2012
Received in revised form 5 October 2012
Accepted 25 October 2012
Available online 30 October 2012

Keywords:
Electrosynthesis
Formatotrophic growth
Biomass yield
Thermodynamic favorability
Chemical motive force
Reductive glycine pathway

ABSTRACT

Electrosynthesis is a promising approach that enables the biological production of commodities, like fuels and fine chemicals, using renewably produced electricity. Several techniques have been proposed to mediate the transfer of electrons from the cathode to living cells. Of these, the electroproduction of formate as a mediator seems especially promising: formate is readily soluble, of low toxicity and can be produced at relatively high efficiency and at reasonable current density. While organisms that are capable of formatotrophic growth, i.e. growth on formate, exist naturally, they are generally less suitable for bulk cultivation and industrial needs. Hence, it may be helpful to engineer a model organism of industrial relevance, such as *E. coli*, for growth on formate. There are numerous metabolic pathways that can potentially support formatotrophic growth. Here we analyze these diverse pathways according to various criteria including biomass yield, thermodynamic favorability, chemical motive force, kinetics and the practical challenges posed by their expression. We find that the reductive glycine pathway, composed of the tetrahydrofolate system, the glycine cleavage system, serine hydroxymethyltransferase and serine deaminase, is a promising candidate to support electrosynthesis in *E. coli*. The approach presented here exemplifies how combining different computational approaches into a systematic analysis methodology provides assistance in redesigning metabolism. This article is part of a Special Issue entitled: Metals in Bioenergetics and Biomimetics Systems.

1. Introduction

In the last decade, the biorefinery concept has become increasingly popular [1–6]. It states that living organisms can and should be used to supply the increasing demand for specialized chemicals, including fuels, solvents, plastics, pharmaceuticals, etc. Today, most of these chemicals are derived, directly or indirectly, from fossil carbons. However, with the imminent depletion of fossil carbons and the concomitant increase in atmospheric CO_2 it has become essential to find alternative sources for these important materials.

The proposed feedstocks for most biorefinery projects are simple sugars, starch, or lingocellulosic biomass [1–6]. While the latter alternative has an apparent advantage by not-competing with human consumption [7,8], it still presents numerous difficulties, including a problematic fermentation technology and challenges in feedstock availability and transportation [9,10]. Electricity is an alternative that can alleviate problems with these bulky feedstocks [11]. Electrons can be shuttled from an electrode to living cells, providing the necessary reducing equivalents and energy to support growth and electrosynthesis of desired commodities [12–16]. Since electricity is widely available, microbial electrosynthesis can be spatially and temporally decoupled from energy production and can take place at any convenient location and time.

Microbial electrosynthesis may be especially useful for the renewable energy market. One major drawback of most renewable energy sources, including solar, wind and hydro-energy, is that their output, i.e. electricity, is difficult to store [17]. Microbial electrosynthesis of fuels could address this problem by efficiently converting electrical energy to kinetically stable chemical bonds.

Several methods of transferring reducing equivalents from an electrode to living cells have been suggested and applied (reviewed in [12–16]). Molecular hydrogen was one of the first electron carriers used as water electrolysis is a relatively mature technology that can support efficient hydrogen production at high current density [18–20]. However, the use of hydrogen suffers from numerous problems including low solubility and the risk of explosion [13]. Moreover, the hydrogenase enzymes that transfer electrons from hydrogen to cellular electron carriers are generally complex, oxygen sensitive proteins, which are hard to express recombinantly [21–24]. As an alternative to molecular hydrogen, several inorganic compounds, such as ferric ion or nitrate, can serve as electron shuttles, supporting electricity-dependent cultivation [16]. However, since the reduction potentials of these compounds are considerably higher than that of NAD(P)H, growth using these substrates requires reverse electron flow [25–29], limiting electrosynthesis to

Abbreviations: $\Delta_r G'$, Transformed Gibbs energy of a reaction; $\Delta_r G'^m$, Transformed Gibbs energy of a reaction under reactant concentrations of 1 mM; THF, Tetrahydrofolate

☆ This article is part of a Special Issue entitled: Metals in Bioenergetics and Biomimetics Systems.

* Corresponding author. Tel.: +972 54 222 4533; fax: +972 8 934 4181.
** Corresponding author. Tel.: +972 50 571 4697; fax: +972 8 934 4181.
E-mail addresses: arren@weizmann.ac.il (A. Bar-Even), ron.milo@weizmann.ac.il (R. Milo).

0005-2728/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbabio.2012.10.013

specific organisms which are less amenable to industrial use. Another option is direct electron transfer from the cathode to microbes. While this approach has several advantages (reviewed in [13,16]), it is limited to a small group of organisms that can associate directly with the cathode or requires complex engineering of others.

As an alternative to the above methods, CO_2 can be directly reduced at the cathode (electrons are derived from water splitting at the anode) [30], providing organic compounds that can be used by living cells as a source of reducing equivalents, energy and carbon. A diverse group of compounds can be produced in this manner [30–33]. The production of simple alcohols such as methanol, ethanol and propanol, hydrocarbons such as methane and ethylene, or acids with more than one carbon such as acetic acid and oxalic acid, has the advantage of supplying microbes with compounds that are relatively simple to metabolize and/or being rich in reducing equivalents. However, the electrocatalytic production of all of these compounds is generally inefficient (not specific to a single product and/or requiring high overpotential), requires costly catalysts and/or supports low current density (reviewed in [30,31]). In contrast, carbon monoxide and formic acid can be produced by direct reduction of CO_2 at relatively high efficiency (although lowering the overpotential needed to reduce CO_2 is still an open challenge) and an acceptable current density [30–37]. Since carbon monoxide is a toxic and flammable gas with low solubility, formic acid, being readily soluble and of low toxicity, is a preferred mediator of electrons. In fact, a formate-based economy was recently proposed as an alternative to the hydrogen-based economy or methanol-based economy concepts [38–41].

Various methylotrophic organisms can grow on formate as a sole carbon, electron and energy source [42–45]. Such organisms can be used for formate-dependent microbial electrosynthesis [43,46]. However, while remarkable progress was achieved in the genetic manipulation, metabolic engineering and bulk cultivation of these organisms, they are still less streamlined for industrial use as compared to model organisms, such as *S. cerevisiae* and *E. coli*. Hence, it can be extremely useful to adopt one of the model organisms, extensively used in the biotechnology industry, for formatotrophic growth (i.e. growth on formate) and electrosynthesis.

This manuscript serves two complementary purposes. First, we investigate the possibility of engineering the industrial model bacterium *E. coli* for formatotrophic growth, paving the way to efficient and sustainable electrosynthesis. Second, to analyze this metabolic possibility, we present, discuss and apply various computational methods that enable a simple comparison between the various pathways that may support formatotrophic growth. We illustrate how these methodologies combine to offer a comprehensive view on a specific metabolic challenge. The details of each of the techniques presented here are described in separate publications (e.g. [47–51]).

2. Formate oxidation supporting autotrophic growth via carbon fixation

There are two parallel strategies to achieve formatotrophic growth of *E. coli*. In the first strategy, the electrons derived from the oxidation of formate to CO_2 are used to support autotrophic growth through CO_2 fixation and ATP supply through respiration; as such, formate molecules are not directly incorporated into cellular metabolism. The second strategy is to use some of the formate molecules to generate energy and reducing power in order to directly assimilate other formate molecules into central metabolism. The first approach is presented in this section, while the second is introduced in the following section.

There are several criteria that should be met in order for a compound to serve as a good electron carrier supplying reducing power for microbial carbon fixation: (i) stability of the donor, both in the reduced and oxidized forms, in order to avoid non-specific reduction of cellular components or fast degradation; (ii) non-toxicity of the donor, both in the reduced and oxidized forms; (iii) permeability of the donor in its reduced and oxidized forms to enable fast electron shuttling without energy investment; (iv) low volatility of the reduced donor, to avoid leakage of reducing power; (v) low reduction potential, such that a direct reduction of NAD(P)H is feasible (reduction potential < -300 mV); (vi) availability of an enzymatic apparatus capable of catalyzing electron transfer from the donor to NAD(P)H and (vii) fast catalysis of electron transfer from the donor to NAD(P)H.

Formate is one of the best electron-mediators, being one of the few compounds that meet almost all of these criteria. In fact, to our knowledge, it is the only electron donor that meets criteria (i) to (vi). Notably, as formate is a very small molecule, it is characterized by a very high permeability coefficient [52,53]. Hence, it can enter the cell through the lipid membrane. Also, a specific formate channel, FocA, facilitates passive transport of formate in and out of the cell [54]. This channel can be overexpressed if formate inward flux becomes limiting.

However, one major challenge remains: the rate of electron transfer from formate to NAD(P) is quite slow. Although fast NAD(P)H-dependent formate dehydrogenases exist that require metal ions for catalysis, these enzymes are extremely oxygen sensitive [55,56] and/or are complex proteins that require unique cofactors [57–60]. As such, they are not suitable for supporting autotrophic growth in a foreign host. On the other hand, simple, metal-free and oxygen-tolerant formate dehydrogenases tend to be relatively slow, with maximal specific activity of ≈ 10 μmol/min/mg [61,62]. For these enzymes to act as a sole supplier of electrons, they must be expressed at high levels and hence impose a considerable protein burden on the cell. Notably, since *E. coli*'s native formate dehydrogenase cannot transfer electrons to NAD(P), it cannot be used to support autotrophic growth and can actually have a deleterious effect by dissipating redox equivalents that otherwise could have been conserved in NAD(P)H [63,64].

Several natural alternative carbon fixation pathways can support autotrophic growth when the cells are supplied with an electron source. These are reviewed and compared extensively elsewhere [49,65–67]. Notably, to the best of our knowledge, the reductive pentose phosphate pathway is the only carbon fixation pathway known to support formate-dependent autotrophic growth in nature (e.g. [68–71]).

Alternatively, by considering the entire set of naturally known metabolic enzymes numerous synthetic carbon fixation pathways can be theoretically constructed [47]. We previously described in detail the MOG pathways, which employ the most effective carboxylating enzymes, PEP and pyruvate carboxylases, and utilize the core of the naturally-evolved C4 cycle. These pathways are predicted to be 2–3 times faster than the reductive pentose phosphate pathway [47].

3. Formate assimilation supporting methylotrophic growth

Instead of oxidizing all formate molecules to CO_2, cells can use some of them as a carbon source, bypassing the need for parallel CO_2 fixation pathways. This formate-dependent methylotrophic alternative is known to occur naturally in various organisms [45,72–75]. The serine pathway is one example for a metabolic route that supports formate assimilation (Fig. 1). In this pathway formate is attached to tetrahydrofolate (THF) at the expense of ATP hydrolysis, forming formyl-THF, which is then reduced to methylene-THF. Methylene-THF donates its formaldehyde moiety to glycine, giving rise to serine, which is converted to glycerate. Glycerate is metabolized to PEP, carboxylated to oxaloacetate and recycled to glycine via malate:CoA ligase and malyl-CoA lyase, yielding acetyl-CoA as the pathway's product (Fig. 1) [45]. In some methylotrophs, acetyl-CoA is then integrated into central metabolism through the unique ethylmalonyl-CoA pathway, such that carbon is finally assimilated on the level of PEP [76–79]. In other methylotrophic bacteria using the serine pathway, acetyl-CoA assimilation takes place through the ubiquitous glyoxylate shunt [80]. If the serine pathway was to be expressed within *E. coli*, this later, endogenous pathway would be used for assimilating acetyl-CoA.

Formate can also be assimilated via the reductive acetyl-CoA pathway found in acetogens [81–84]. In this pathway methylene-THF is

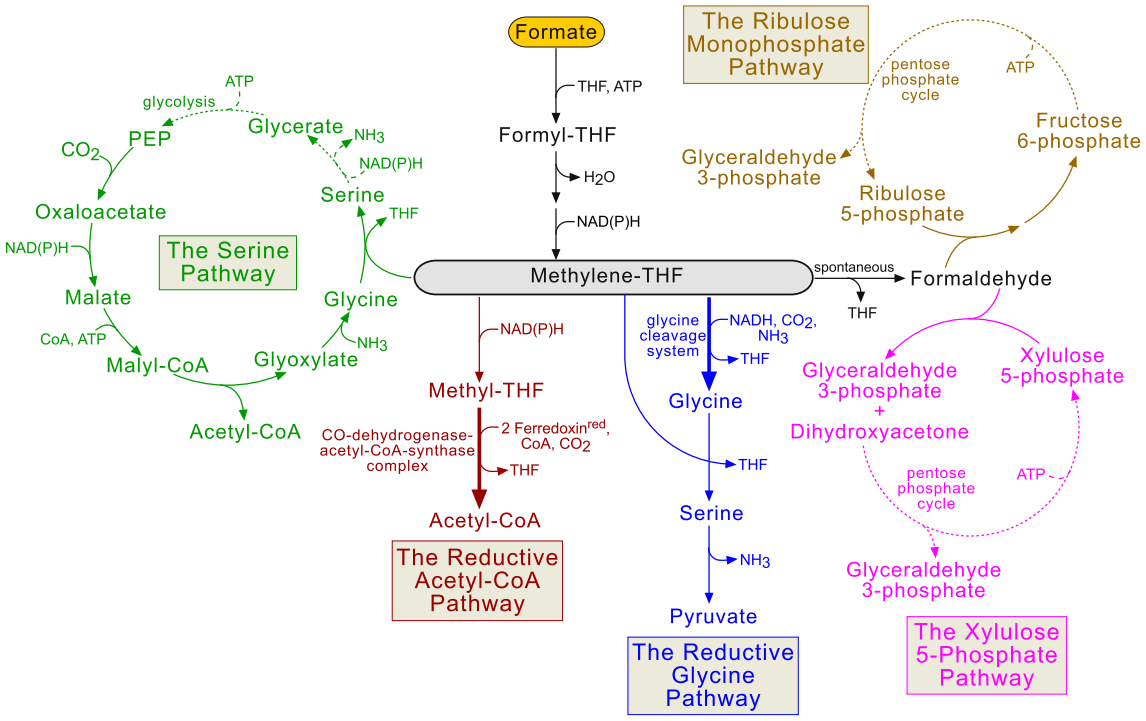

Fig. 1. Formate-assimilating pathways. All pathways start from formate (marked with yellow) attached to tetrahydrofolate (THF) and reduced to methylene-THF. The serine pathway (shown in green) and the reductive acetyl-CoA pathway (maroon) are known to support formatotrophic growth in various organisms. The ribulose monophosphate pathway (shown in brown) and the xylulose 5-phosphate pathway (purple) are known to support methylotrophic growth but there is no indication that they support formatotrophic growth. They might be able to do so through the cleavage of metheylene-THF to formaldehyde and THF. The reductive glycine pathway (shown in blue) is not known to support formatotrophic (or methylotrophic) growth but can do so in principle. Solid arrows correspond to single enzymes. Dashed arrows correspond to the sequential operation of several enzymes. Bold arrows correspond to several enzymes operating within a complex.

further reduced to methyl-THF. Methyl-THF donates its C1 moiety to CO, produced by CO dehydrogenase, that together with CoA give rise to acetyl-CoA (Fig. 1).

It is possible that formate can also be assimilated through the ribulose monophosphate pathway or via the xylulose 5-phosphate pathway of methylotrophic yeasts (Fig. 1) [42,45,85]. These pathways react a pentose phosphate with a free formaldehyde to produce C6 or C3 central metabolic compounds which can then recycle the initial reactant through the pentose phosphate cycle [42,45,85]. Free formaldehyde can potentially be produced via a spontaneous hydrolysis of methylene-THF to THF and formaldehyde (Fig. 1).

As in the case of carbon fixation [47], synthetic formate-assimilating pathways can be computationally suggested by considering the entire set of naturally known metabolic enzymes. We used the constraint-based modeling approach described in [47] to systematically search for formate assimilating pathways. We discarded all pathways containing thermodynamically unfavorable reaction sequences [47,48,50,67].

Regardless of the exact conditions applied or the identity of the pathway product, our algorithm consistently identified one short, favorable pathway. This pathway, composed of the tetrahydrofolate system, the glycine cleavage system, serine hydroxymethyltransferase and serine deaminase, was termed the reductive glycine pathway (Fig. 1). A similar pathway was previously found to serve as an electron sink recycling reduced electron carriers that are generated during the fermentation of purines and amino acids in some bacteria (the reductive glycine pathway differs from that pathway in bypassing the selenium- and thioredoxin-dependent glycine reductase and using serine hydroxymethyltransferase and serine deaminase instead) [86–93]. Recently, it was also suggested that the reductive glycine pathway (without the terminal serine deaminase) is a common metabolic route for glycine and serine synthesis in various prokaryotes, including autotrophic ones [94]. The pathway was further suggested to operate in the last universal common ancestor (LUCA) [94]. However, there is no indication that the reductive glycine pathway, on its own, supports methylotrophic or autotrophic growth in any organism. Notably, the glycine cleavage system, the 'core' of the reductive glycine pathway, was shown to be fully reversible, *in-vitro* and *in-vivo*, also in organisms that do not depend on it for glycine production, such as *Saccharomyces cerevisiae*, animal and plant mitochondria (e.g. [95–100]).

In all the different pathways, natural and synthetic, the assimilation of formate proceeds via its ligation with THF and requires reduction to methylene-THF to match the average oxidation state of cell carbon. However, the reduction of a carboxyl (formate) is unfavorable if unactivated [67]. Carbon fixation pathways activate this reaction by an ATP-dependent attachment of a phosphate or a CoA moiety to the carboxyl. In formate assimilation pathways, the ATP invested in the ligation of formate with THF serves to energize this reduction, as discussed in detail in [67]. Hence, the THF system provides a thermodynamically favorable route to assimilate formate into the reduced environment of the cell.

4. Biomass yield for growth on formate

In order to analyze which strategy (and corresponding pathways) might be most suitable to support growth on formate we compared them using several criteria [47]. First, we asked what is the expected biomass yield on formate for each of the different pathways [45,51,72,101].

Before applying quantitative methods to address the biomass yield of the different pathways, a qualitative estimation can be made based on our knowledge of the energetic profile of biochemical reactions [67]. The sum of formate molecules required either as carbon source or as reducing source is fixed regardless of the exact pathway or metabolic strategy. For example, five formate molecules are required as carbon and reducing power sources to produce pyruvate, either via a carbon fixation pathway (five formate molecules are required for reducing power)

or a formate assimilation route (e.g. two formate molecules for reducing power and three as carbon source).

However, the number of formate molecules needed to be invested in ATP production is strongly affected by the metabolic strategy chosen. Carbon fixation pathways need to energize unfavorable carboxylations and carboxyl reductions by hydrolyzing ATP (e.g. acetyl-CoA carboxylase, succinyl-CoA reductase, pyruvate synthase, etc. [67]). In contrast, directly assimilating formate bypasses ATP-dependent carboxylations (although ATP is still required for carboxyl reduction activation) [67]. We therefore expect formate assimilating pathways to exhibit higher biomass yield than their carbon fixation counterparts since they need to invest less formate molecules for ATP production. Yet, some carbon fixation pathways, such as the reductive TCA cycle, couple carboxylation reactions to exergonic reactions (reactions which lead to a decrease in Gibbs free energy) other than ATP hydrolysis, thereby having low ATP requirement [67]. These pathways are expected to support high biomass yield as well.

We used two quantitative methods to estimate the biomass yield on formate of each of the pathways. The "carbon-source-conversion" method [45] is a back-of-the-envelope approach for estimating biomass yield — less rigorous but easy to implement. In this method, we first calculate the yield of converting formate into a designated benchmark metabolite, $Y_{\text{formate->metabolite}}$, in units of mol/mol, considering the formate molecules that served as a carbon source and those that were oxidized to provide the electrons and ATP consumed during the conversion of formate to the benchmark metabolite. Using experimentally measured biomass yield for growth on that metabolite, $Y_{\text{metabolite->biomass}}$, in units of gCDW/mol (CDW being cellular dry weight), we can then estimate the biomass yield on formate, in units of gCDW/mol formate, as $Y_{\text{formate->biomass}} = Y_{\text{formate->metabolite}} \cdot Y_{\text{metabolite->biomass}}$.

For example, we calculated the number of formate molecules needed to be invested to generate one molecule of pyruvate. The reciprocal of this number is the pyruvate yield on formate in units of mol pyruvate/mol formate. Multiplying pyruvate yield on formate with biomass yield on pyruvate, ≈15 gCDW/mol-pyruvate, as measured experimentally [102], provides us with an estimate of the biomass yield on formate in units of gCDW/mol formate. The same process was then repeated for glucose and acetate, using the experimentally measured biomass yields on glucose, ≈71 gCDW/mol-glucose and acetate, ≈23 gCDW/mol-acetate [102]. We assumed that ATP is produced from NADH through oxidative phosphorylation and that the P/O ratio (measuring how many ATP molecules are produced per one oxygen atom being reduced) is 1.5, as is relevant for *E. coli* [103]. The second, third and fourth columns of Table 1 display the estimated biomass yield calculated by choosing pyruvate, glucose or acetate as the product of the carbon fixation and formate-assimilating pathways. Notably, in most of the pathways, less formate molecules are invested for the generation of ATP than those invested for the production of reducing equivalents or used as a carbon source.

The results shown in Table 1 suggest that this type of analysis is problematic since the choice of the specific metabolite to serve as the product of the pathways can influence the estimation greatly: assuming glucose or acetate as the pathways' product gave much higher estimation for biomass yield on formate simply because the biomass yield of *E. coli* on glucose and acetate is higher than that on pyruvate.

An alternative approach to estimate biomass yield is to use flux balance analysis [104,105] as implemented in [51]. The advantage of this approach is that it makes no *a priori* assumptions about which metabolites formate will be converted into in order to serve as a carbon source. Rather, this approach treats the entire cellular biomass as the product of carbon fixation or formate assimilation [51]. We chose to use the core *E. coli* metabolic model (including glycolysis, pentose phosphate cycle, TCA cycle, glyoxylate shunt, respiration, etc.) [106] over the full metabolic model since we wanted our analysis to be somewhat general, i.e. not overly specific to *E. coli*, and since we wanted to avoid improbable metabolic pathways that sometimes emerge when using the full metabolic model. It is important to note, however, that the biomass compositions of organisms can vary, and hence the results obtained using the core *E. coli* metabolic model might not be suitable for all organisms [107].

We used flux balance analysis to calculate growth yield rather than growth rate. Hence, formate input units were in mmol and biomass yield was given in gDW. Since the ATP maintenance cannot be estimated *a priori* for growth on formate we calculated the biomass yields in two different ways, representing two extremes: once removing ATP maintenance altogether and once assuming an ATP maintenance requirement identical to that of growth on glucose. The fifth and sixth columns in Table 1 display the results of this analysis.

Interestingly, the measured biomass yield for growth on formate using the serine pathways is 3.3-6.9 gCDW/mol-formate [72], which matches well the estimated biomass yield based on the flux balance analysis with no ATP-maintenance and on the "carbon-source-conversion" method, assuming acetate as the product of formate assimilation (5.5 and 4.4 gCDW/mol-formate, respectively, see Table 1). The measured biomass yield for growth on formate using the reductive pentose phosphate cycle is 2–4 gCDW/mol-formate [69,70], fitting the estimation produced by the "carbon-source-conversion" method, assuming glucose or acetate as the products of carbon fixation, and by the flux balance analysis with ATP maintenance identical to that of growth on glucose.

Importantly, while the methods of calculating biomass yield on formate differ substantially and resulted in up to 4 fold difference in estimated biomass yield (Table 1), they suggested very similar relative biomass yields of the pathways. In fact, the correlation between the biomass yields calculated by assuming pyruvate, glucose or acetate as the pathways' product and those calculated using flux balance analysis has $R^2 > 0.55$. Considering all the metrics discussed, the initial qualitative expectation is generally confirmed: formate-assimilating pathways support higher biomass yield than carbon fixation ones. The exceptions to this rule are the reductive TCA cycle that supports very high biomass yield due to a very low ATP requirement and the serine pathway that is expected to support low biomass yield, at least as compared to other formate assimilation pathways.

5. Thermodynamic favorability

Not all metabolic pathways which operate in one organism will be thermodynamically favorable if expressed in other organisms, where the cellular conditions (pH, ionic strength, etc.) might differ considerably [47,67,108]. We therefore checked whether the pathways discussed above are thermodynamically favorable in the conditions prevailing in *E. coli*'s cytosol (pH ≈ 7.5, ionic strength ≈ 0.2 M). We tested not only the favorability of the pathway net reaction [47,49] but also analyzed whether the pathway contained distributed thermodynamic bottlenecks composed of several reactions [47,109].

Notably, the CO-dehydrogenase-acetyl-CoA-synthase and the glycine-cleavage-system are complexes of several enzymes. Reactions occurring within complexes are, in many cases, coupled to each other, overcoming any internal thermodynamic barriers [67]. Indeed, within the CO-dehydrogenase-acetyl-CoA-synthase complex CO appears to be a bound intermediate, which is shuttled ≈70 Å within an internal tunnel from the CO-dehydrogenase cluster to that of acetyl-CoA synthase [110–112]. This means that even if the complex is expressed within a foreign host, the reduction of CO_2 will probably be coupled to acetyl-CoA synthesis, hence diminishing any internal barriers. This coupling is suggested to be very important in the case of the reductive acetyl-CoA pathway. The reduction of CO_2 to CO with ferredoxin as electron donor is highly unfavorable ($\Delta_r G'^m > 30$ kJ/mol) and cannot take place unless directly coupled with the very favorable acetyl-CoA synthase reaction. The overall reaction of this complex, methyl-THF + CO_2 + 2 ferredoxin^{red} + CoA → acetyl-CoA + 2 ferredoxin^{ox} + THF, has $\Delta_r G'^m < 0$, making the net complex reaction favorable and enabling pathway operation. Hence, for the sake of thermodynamic analysis, all reactions taking

Table 1
Some properties of the pathways capable of supporting formatotrophic growth.

Pathway	Biomass yield, gCDW/mole-Formate					Optimized Bottleneck Energetics (OBE), kJ/mol [#]	Number of foreign enzymes [##]
	Assuming formate is first converted to… [*]			Flux-balance-analysis [**]			
	pyruvate	glucose	acetate	With ATP maintenance [***]	With no ATP maintenance		
Carbon fixation pathways							
reductive pentose phosphate	1.5	3.0	2.9	2.5	5.6	9.4	3
reductive TCA	2.3	3.8	5.0	2.9	6.7	Infeasible	4
dicarboxylate-4-hydroxypropionate	1.8	3.1	3.5	2.4	5.3	4.2	5
3-hydroxypropionate-4-hydroxybutyrate	1.3	2.5	3.2	1.8	4.2	4.2	>5
3-hydroxypropionate	1.5	2.8	2.9	1.9	4.4	6.5	>5
MOG [47]	1.6	3.1	3.2	2	4.5	5.3	>5
Formate assimilating pathways							
reductive acetyl-CoA	2.6	4.1	5.9	2.7	6.2	6.5	4
serine	1.6	3.0	4.4	2.4	5.5	6.2	5
ribulose monophosphate	2.1	3.8	4.4	2.8	6.4	3.3	4
xylulose 5-phosphate	1.9	3.5	3.9	2.6	5.9	8.1	3
Reductive glycine	2.3	3.8	5.0	2.8	6.3	5.4	2

[*] The Carbon-Source-Conversion method as described in the main text.
[**] We used the core *E. coli* metabolic model [106] and ran flux-balance-analysis with the following constraints: formate input of 10 mmol, unrestricted oxygen and CO_2 input and output, no flux through pyruvate formate lyase and no input of any other carbon source. Biomass objective function was taken as in [106].
[***] ATP maintenance was taken as 8.39 mmol, identical to the value used for growth on glucose, as given in the core metabolic model [106].
[#] The Optimized Bottleneck Driving-Force (OBD) is $-\Delta_rG'$ of the distributed bottleneck reactions, after the reactant concentrations have been optimized, see main text. Reactions within complexes were considered as a single overall net reaction. The reductive TCA cycle is infeasible under *E. coli*'s physiological conditions.
[##] Number of foreign enzymes that need to be expressed in wild type *E. coli* to establish an active pathway.

place in a complex were considered to be coupled and hence represented by a single overall net reaction.

We found only one pathway that is predicted to be thermodynamically unfavorable at the cellular conditions of *E. coli* and under physiological reactant concentrations (1 μM-10 mM for non co-factor metabolites [50,67,113,114]): the reductive TCA cycle. This pathway is expected to be infeasible due to the sequential operation of 2-ketoglutarate synthase and isocitrate dehydrogenase. Each of these enzymes is moderately unfavorable but can still carry flux in the reductive direction if reactant concentrations are changed within the physiological range. However, the sequential operation of the two enzymes creates an energetic barrier that cannot be overcome by modulating reactant concentrations within the physiological range we consider for *E. coli*.

These two enzymes catalyze the overall reaction succinyl-CoA + 2 $ferredoxin^{red}$ + NADPH + 2 CO_2 → isocitrate + CoA + 2 $ferredoxin^{ox}$ + $NADP^+$. Even if we assume that the concentration of dissolved CO_2 is kept at 1 mM (≈100 fold higher then ambient CO_2 concentration), [NADPH] = 10·[$NADP^+$], [$ferredoxin^{red}$] ≈ [$ferredoxin^{ox}$], [CoA] = 1 mM (the lowest cellular concentration of this cofactor in *E. coli* [113]) and the other reactants at extreme concentrations with [succinyl-CoA] = 10 mM and [isocitrate] = 1 μM, Δ_rG' is still positive at pH 7.5 and ionic strength of 0.2 M [48]. This likely rules out the reductive TCA cycle as a potential carbon fixation pathway in an engineered *E. coli* strain. Notably, the reductive TCA cycle can operate in other organisms since the cytosolic conditions might be considerably different, e.g. pH<7, [CoA]<< 1 mM and/or very high [CO_2] due to carbon concentrating mechanisms.

6. Chemical motive force

Thermodynamic favorability is insufficient for efficient pathway operation as the energy dissipated during a reaction can have a substantial effect on its kinetics. In fact, $\Delta G_r'$ dictates what fraction of the enzymatic machinery catalyzes the forward reaction according to the force-flux relationship [49,115–117]: $\Delta G_r' = -RT\ln(J^+/J^-)$, where J^+ is the forward flux, J^- is the backward flux, R is the gas constant and T is the temperature in Kelvin. Hence, a low (negative) $\Delta G_r'$ value, corresponding to a high chemical motive force, indicates that most of the enzymatic machinery is catalyzing the forward reaction and hence a high metabolic rate can be achieved. Assuming substrate saturation and similar kinetics in the forward and backward direction, $\Delta G_r'$ of −7.5 kJ/mol corresponds to a reaction that proceeds at 90% of its maximal velocity: 95% of the enzymes catalyze the forward reaction while 5% catalyze the backward reaction.

We asked which of the pathways analyzed can, in principle, support high flux in terms of the chemical motive force sustained by each of its reactions. To address this question we define the distributed bottleneck of a pathway, as the reaction(s) with the lowest $-\Delta_rG'$ (i.e. closest to zero) under given reactant concentrations. The Optimized Bottleneck Driving-Force (OBD) is defined as the $-\Delta_rG'$ of the distributed bottleneck reaction(s), after optimizing reactant concentrations (within the physiological range) in order to increase it as much as possible (the details of the OBD analysis are to be published in a separate paper soon). A high OBD corresponds to high chemical motive force and hence high rate throughout all pathway reactions. Low OBD corresponds to a significant backward flux and hence decreased rate. The seventh column in Table 1 displays the OBD calculated for all the carbon fixation and formate assimilation pathways. It is clear that some pathways are considerably more constrained than others. For example, the reductive pentose phosphate pathway has an OBD almost three times higher than that of the ribulose monophosphate pathway, which is expected to have a considerable backward flux, reducing the net pathway rate.

7. Pathway kinetics

The kinetic parameters, i.e. maximal velocity (V_{MAX}) and affinities toward the substrates (Michaelis constants, K_M), are at least as important as the chemical motive force in determining reaction rate. In fact, the chemical motive force and the enzymatic parameters can compensate for each other: a low fraction of enzymes catalyzing the forward reaction might still enable high flux by virtue of a high maximal velocity, while, conversely, a low maximal velocity can sometimes be tolerated if backward flux is minimal. Thus, it would be beneficial to use a single framework that estimates pathway kinetics by considering both the chemical motive force and the enzymatic parameters. Unfortunately, such a framework is currently unavailable.

In a previous study we suggested and utilized a criterion termed the 'pathway specific activity' that serves as a simple upper limit proxy for pathway kinetics based on the maximal velocities of each of its enzymes (but not considering the chemical motive force). Analysis of the pathway specific activities of the carbon fixation pathways

is given in refs. [47,49]. Unfortunately, this criterion is not very useful for the formate-assimilating pathways for several reasons. First, the kinetics of the reactions within the CO-dehydrogenase-acetyl-CoA-synthase and the glycine-cleavage-system complexes cannot be inferred with sufficient accuracy from the kinetics of the purified enzymatic components. Specifically, the kinetics within complexes is expected to be substantially better as compared to that of the isolated reactions. Second, two of the formate-assimilating pathways are based on the spontaneous cleavage of methylene-THF to formaldehyde and THF, which cannot be incorporated into the pathway specific activity metric as currently defined.

Yet, some back-of-the-envelope calculations can shed light on the usability of some of the candidate pathways. For example, we can estimate the maximal growth rate for a bacterium utilizing formate solely as an electron source and relying on carbon fixation pathways for carbon. We consider the growth rate limit imposed by the rate of formate dehydrogenase, since, as described above, its slow rate might limit redox and energy supply to the cell.

Assuming that cells express a formate dehydrogenase with a specific activity of 10 μmol/min/mg (maximal velocity requiring the simple formate dehydrogenase variants, see above) at 20% of its total protein (more than that is expected to impose a very high burden on the cells and hence limit growth). If ≈50% of the cellular dry weight is proteins [118] we get that ≈10% of the cellular dry weight is formate dehydrogenase and hence the specific activity is ≈1 μmol/min/mgCDW. If the reductive pentose phosphate pathway serves as the carbon fixation pathway, 12 NAD(P)H and 18 ATP molecules are required to fix six CO_2 molecules to glucose. Assuming a P/O ratio of 1.5 [103], 12 + 18/1.5 = 24 molecules of formate should be oxidized to support the formation of one glucose molecule. Hence, the rate of glucose formation will be 1/24 of the specific activity above, ≈0.04 μmol-Glucose/min/mgCDW. Since the experimentally measured biomass yield on glucose is ≈71 gDW/mol [102] we get this rate to equal $0.04 \cdot 71 \cdot 10^{-6} \approx 3 \cdot 10^{-6}$ gCDW/min/mgCDW or 0.003 mgCDW/min/mgCDW. Hence, the growth rate equals 0.003 ≈ 1/min and the doubling time is ln(2)/0.003 ≈ 230 min ≈ 4 hours. Repeating the same calculation but assuming that pyruvate or acetate is the product of formate assimilation results in a doubling time of ≈7.5 hours or ≈4 hours, respectively.

This calculation suggests that the doubling time of an autotrophic bacterium metabolizing formate using a formate dehydrogenase of the 'simple' kind cannot be lower than 4 hours. Of course, this is only an optimistic estimate and the doubling time might very well be limited by other factors, such as the rate of carbon fixation. In addition, even the rough limit of 4 hours can change when considering carbon fixation pathways with different ATP requirements or if the expression of formate dehydrogenase is restricted to be below 20% of total protein. On the other hand, organisms that employ the more 'elaborate' formate dehydrogenase enzymes, having significantly higher specific activities, can sustain doubling time faster than 4 hours, as supported by experimental measurements [70,71,119].

A similar analysis can produce more conclusive results. For example, we ask whether a bacterium can use the ribulose monophosphate or the xylulose 5-phosphate pathways for formate assimilation when limited by the rate of spontaneous cleavage of methylene-THF to formaldehyde and THF. According to the equilibrium and kinetic constants reported in [120] and assuming a high [methylene-THF] ≈ 10 mM, the maximal rate of methylene-THF cleavage can be calculated to be ≈0.03 mM/sec. Since the cellular volume of slowly growing *E. coli* is ≈1 μm^3 [121] this rate equals $\approx 3 \cdot 10^{-17}$ mmol/sec/cell or $\approx 2 \cdot 10^{-12}$ μmol/min/cell. Since six such reactions are required for the production of one glucose molecule the rate of glucose production is limited to $2 \cdot 10^{-12}/6 \approx 3 \cdot 10^{-13}$ μmol-Glucose/min/cell. The dry weight of an *E. coli* cell with a volume of ≈1 μm^3 is ≈200 fg [122] and hence the former rate equals ≈0.0015 μmol-Glucose/min/mgCDW. According to the above biomass yield this rate corresponds to $\approx 10^{-7}$ gCDW/min/mgCDW which is 10^{-4} mgCDW/min/mgCDW. The growth rate therefore equals 10^{-4} 1/min and the doubling time is $\ln(2)/10^{-4} \approx 7000$ min ≈ 120 hours = 5 days.

Therefore, a simple back-of-the-envelope calculation, considering the best-possible case of a single rate-limiting reaction, suggests that two of the formate-assimilating pathways are kinetically inefficient and can support only a very slow growth, which, in many cases, has little physiological meaning. Indeed, a previous study has demonstrated that the reverse reaction — the spontaneous condensation of THF with formaldehyde — is too slow to have any metabolic significance *in vivo* [123].

8. Pathway expression challenges

Finally, we asked how challenging we expect the heterologous expression of an active pathway in a new host (*E. coli* in our case) to be. One aspect that affects the expression difficulty is the number of foreign enzymes that must be expressed to enable pathway activity. The rightmost column in Table 1 displays this number—relative to wild type *E. coli* — for each of the metabolic alternatives. All approaches require, or strongly benefit from, the expression of formate dehydrogenase to supply the cell with reducing power and energy. Importantly, formate dehydrogenases of the simple, metal-free type were shown to be fully active when expressed in *E. coli* (reviewed in ref. [62]).

The reductive glycine pathway presents the smallest expression barrier: only foreign formate dehydrogenase and formate-tetrahydrofolate ligase are needed for pathway operation. The reductive pentose phosphate pathway and the xylulose 5-phosphate pathway also seem to impose a small barrier, requiring only three foreign enzymes each.

There are several other aspects that might hinder the expression of an active pathway, including how many genes are required to encode each of the enzymes, whether or not the enzymes work in a complex, whether unique co-factors are required by some enzymes and the specific conditions the enzymes are restricted to. In fact, some of the pathways discussed here employ enzymes that were never successfully expressed in a foreign host and some necessitate the expression of further proteins to enable correct folding and activation of the pathway enzymes.

In this manuscript we cannot cover all aspects for all pathways. However, we mention two important issues briefly. First, some of the enzymes operating in some of the pathways are sensitive toward molecular oxygen [49,65]. Specifically, the reductive TCA cycle and the dicarboxylate-4-hydroxypropionate pathways employ several oxygen sensitive enzymes [49,65]. Also, the reductive acetyl-CoA pathway operates some of the most oxygen sensitive enzymes known [49,65,81]. These pathways are therefore not suitable if the bacterium is to be cultivated under aerobic conditions. Second, some pathways involve intermediate metabolites of considerable toxicity to the cell. Specifically, the ribulose monophosphate and xylulose 5-phosphate pathways require the presence of free formaldehyde as an intermediate. Yet, formaldehyde is a toxic compound that can react with various molecules within the cell (e.g. [124]). Indeed, *E. coli* has evolved multiple detoxifying mechanisms to relieve formaldehyde toxicity [125]. Therefore, operating pathways that involve the formation of free formaldehyde raises special challenges.

9. The reductive glycine pathway: a promising formate assimilation pathway

Discarding all the pathways that (i) support relatively low biomass yield (most carbon fixation pathways and the serine pathway); (ii) are thermodynamically infeasible (TCA cycle); (iii) are predicted to operate under low chemical motive force (the 4-hydroxybutyrate carbon fixation pathways and the ribulose monophosphate pathway); (vi) contain a major kinetic bottleneck (ribulose monophosphate and xylulose 5-phosphate pathways) and (v) are expected to present severe expression problems (reductive acetyl-CoA pathway), we are left with a single pathway. The reductive glycine pathway, shown in detail in Fig. 2,

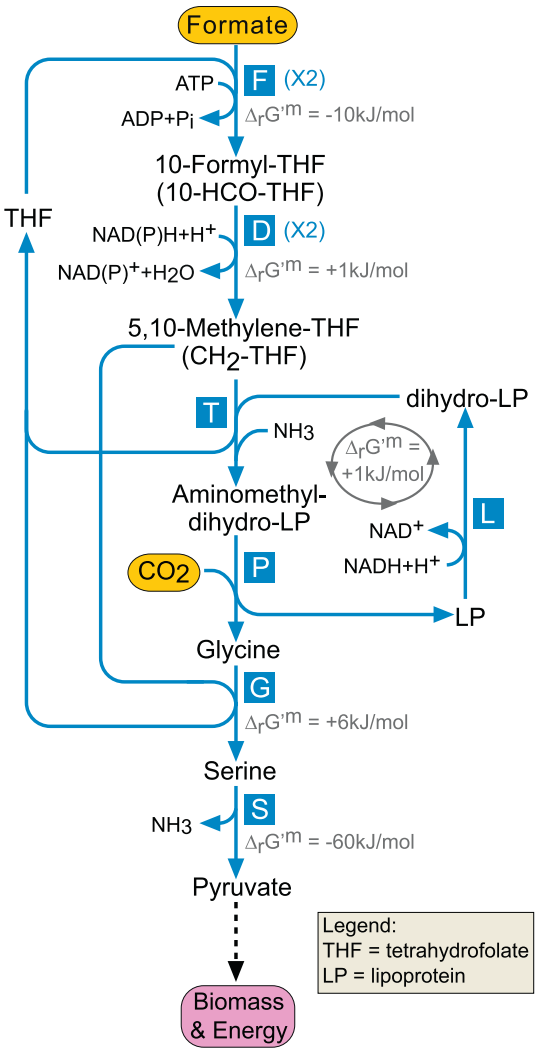

Fig. 2. The proposed reductive glycine pathway. The pathway reduces and condenses two formate molecules and one CO_2 molecule to form pyruvate. $\Delta_rG'^m$ is the reaction change in Gibbs energy when all reactants are at a concentration of 1 mM (pH 7). Enzyme abbreviations: 'F' corresponds to formate tetrahydrofolate ligase (EC 6.3.4.3), 'D' to bifunctional methenyltetrahydrofolate cyclohydrolase/dehydrogenase (EC 3.5.4.9 and 1.5.1.5/15), 'T' to aminomethyltransferase (EC 2.1.2.10), 'P' to glycine dehydrogenase (decarboxylating) (EC 1.4.4.2), 'L' to dihydrolipoyl dehydrogenase (EC 1.8.1.4), 'G' to serine hydroxymethyltransferase (EC 2.1.2.1) and 'S' to serine deaminase (EC 4.3.1.17).

requires the expression of only two foreign enzymes (considering *E. coli* as the host), contains no oxygen sensitive enzyme, supports high biomass yield and is able to sustain a reasonably high chemical motive force through the entire reaction set (Table 1). This pathway seems to be a promising route to establish a formatotrophic *E. coli*.

Yet, this pathway comes with its own challenges. The main difficulty associated with this pathway is the operation of *E. coli*'s glycine cleavage system in the reductive, glycine forming direction. While it was never demonstrated that *E. coli*'s glycine cleavage system can actually produce glycine *in-vivo*, evidence from several parallel studies suggest that such a reaction can take place. Yet, the kinetics of the system might be limited by the relatively low affinities toward CO_2 and ammonia and by the rate of the carboxylation reaction [126–130] (though since these parameters were measured for the components of the system separately and not within the complex, it is not clear how relevant they are.) Hence, for the proper operation of the pathway it will be important to first cultivate the bacterium under high CO_2 and ammonia concentrations. Once formatotrophic growth is established, adaptation to lower CO_2 and ammonia concentrations can take place. Expressing an efficient glycine cleavage system from another organism can also enhance pathway kinetics, although this might impose further expression challenges.

10. Concluding remarks

Some of the pathways we analyzed have several variants, each with its own characteristics, advantages and drawbacks. While we do not have the space to consider all of these alternatives in this manuscript, they should be taken into consideration. Some pathway variants might be substantially better than others. For example, the reductive TCA cycle is probably not a good candidate since it contains a huge thermodynamic barrier at *E. coli*' s cellular conditions. However, *Hydrogenobacter thermophilus* employs an ATP-dependent mechanism to push the pathway in the reductive direction: the enzyme 2-ketoglutarate carboxylase catalyzes the ATP-dependent carboxylation of 2-ketoglutarate to oxalosuccinate using a biotin-dependent mechanism, while oxalosuccinate is further reduced to isocitrate by a non-carboxylating isocitrate dehydrogenase [131,132]. This pathway variant is thermodynamically favorable and even supports high chemical motive force for each of its enzymatic components. However, the soluble intermediate oxalosuccinate is unstable and readily undergoes decarboxylation, creating a futile cycle that reduces the overall efficiency of carbon fixation [131].

In addition, laboratory enzyme evolution can offer solutions to many of the challenges we have raised. For example, the enzyme catalyzing the reversible condensation of formaldehyde and tetrahydromethanopterin to methylene-tetrahydromethanopterin [133] might be evolved to accept THF instead of tetrahydromethanopterin, thereby lifting the kinetic barrier imposed by the spontaneous cleavage of methylene-THF (although the problem of formaldehyde toxicity still remains). However, since the physicochemical properties of tetrahydromethanopterin are quite different from those of THF [94], such enzyme evolution might pose considerable challenges.

Of special interest is the design of an enzyme that can condense two formate molecules to glyoxylate which can then be directly assimilated into central metabolism. While such an enzyme was previously suggested to operate in the chloroplast of greening potato tubers [134–136], the energetics of this unactivated condensation is extremely unfavorable, indicating that the report is probably erroneous [49]. Any attempt to design an enzyme that catalyzes this condensation must therefore first activate the formate (with a phosphate group, for example).

Beyond the issue of the pathways eventually be used to support formatotrophic growth of *E. coli*, we hope that the criteria summarized and reviewed in this manuscript can provide a useful toolbox for other metabolic engineering projects.

Acknowledgments

We thank Igor Bogorad, Rogier Braakman and Justin Siegel for helpful discussions and critique reading of the manuscript. E.N. is grateful to the Azrieli Foundation for the award of an Azrieli Fellowship. This study was supported by the European Research Council (grant 260392-SYMPAC), Israel Science Foundation (grant 750/09), Kahn center for systems biology and the Helmsley program on Alternative Energy at the Weizmann Institute of Science. R.M is the incumbent of the Anna and Maurice Boukstein career development chair.

References

[1] S. Fernando, S. Adhikari, C. Chandrapal, N. Murali, Biorefineries: current status, challenges, and future direction, Energy Fuels 20 (2006) 1727–1737.
[2] B. Kamm, M. Kamm, Principles of biorefineries, Appl. Microbiol. Biotechnol. 64 (2004) 137–145.
[3] B. Kamm, P.R. Gruber, M. Kamm, Biorefineries — Industrial Processes and Products, Wiley-VCH Verlag GmbH & Co. KGaA, 2002.
[4] B. Kamm, M. Kamm, Biorefineries–multi product processes, Adv. Biochem. Eng. Biotechnol. 105 (2007) 175–204.
[5] A. Demirbasm, Biorefineries: current activities and future developments, Energy Convers. Manag. 50 (2009) 2782–2801.

[6] J.H. Clark, F.E.I. Deswarte, T.J. Farmer, The integration of green chemistry into future biorefineries, Biofuels, Bioprod. Biorefin. 3 (2009) 72–90.
[7] D. Pimentel, A. Marklein, M.A. Toth, M.N. Karpoff, G.S. Paul, R. McCormack, J. Kyriazis, T. Krueger, Food Versus Biofuels: Environmental and Economic Costs, Hum. Ecol. 37 (2009) 1–12.
[8] A.J. Wargacki, E. Leonard, M.N. Win, D.D. Regitsky, C.N. Santos, P.B. Kim, S.R. Cooper, R.M. Raisner, A. Herman, A.B. Sivitz, A. Lakshmanaswamy, Y. Kashiyama, D. Baker, Y. Yoshikuni, An engineered microbial platform for direct biofuel production from brown macroalgae, Science 335 (2012) 308–313.
[9] C. Weber, A. Farwick, F. Benisch, D. Brat, H. Dietz, T. Subtil, E. Boles, Trends and challenges in the microbial production of lignocellulosic bioalcohol fuels, Appl. Microbiol. Biotechnol. 87 (2010) 1303–1315.
[10] A. Singh, D. Pant, N.E. Korres, A.S. Nizami, S. Prasad, J.D. Murphy, Key issues in life cycle assessment of ethanol production from lignocellulosic biomass: challenges and perspectives, Bioresour. Technol. 101 (2011) 5003–5012.
[11] R.J. Conrado, C.A. Haynes, B.E. Haendler, E.J. Toone, Electrofuels: a new paradigm for renewable fuels, in: J.W. Lee (Ed.), Advanced Biofuels and Bioproducts, 2012, pp. 1037–1064.
[12] K. Rabaey, R.A. Rozendal, Microbial electrosynthesis — revisiting the electrical route for microbial production, Nat. Rev. Microbiol. 8 (2010) 706–716.
[13] D.R. Lovley, Powering microbes with electricity: direct electron transfer from electrodes to microbes, Environ. Microbiol. Rep. 3 (2011) 27–35.
[14] D.R. Lovley, K.P. Nevin, A shift in the current: new applications and concepts for microbe–electrode electron exchange, Curr. Opin. Biotechnol. 22 (2011) 441–448.
[15] K. Rabaey, P. Girguis, L.K. Nielsen, Metabolic and practical considerations on microbial electrosynthesis, Curr. Opin. Biotechnol. 22 (2011) 371–377.
[16] J.C. Thrash, J.D. Coates, Review: direct and indirect electrical stimulation of microbial metabolism, Environ. Sci. Technol. 42 (2008) 3921–3931.
[17] M. Nicholson, Storing electricity, in: The Power Makers' Challenge, 2012.
[18] J. Turner, G. Sverdrup, M.K. Mann, P.C. Maness, B. Kroposki, M. Ghirardi, R.J. Evans, D. Blake, Renewable hydrogen production, Int. J. Energy Res. 32 (2008) 379–407.
[19] L.M. Gandía, R. Oroz, A. Ursúa, P. Sanchis, P.M. Diéguez, Renewable hydrogen production: performance of an alkaline water electrolyzer working under emulated wind conditions, Energy Fuel 21 (2007) 1699–1706.
[20] P. Milleta, D. Dragoea, S. Grigorievb, V. Fateevb, C. Etievantc, GenHyPEM: a research program on PEM water electrolysis supported by the European Commission, Int. J. Hydrogen Energy 34 (2009) 4974–4982.
[21] D.W. Mulder, E.M. Shepard, J.E. Meuser, N. Joshi, P.W. King, M.C. Posewitz, J.B. Broderick, J.W. Peters, Insights into [FeFe]-hydrogenase structure, mechanism, and maturation, Structure 19 (2011) 1038–1052.
[22] M.J. Corr, J.A. Murphy, Evolution in the understanding of [Fe]-hydrogenase, Chem. Soc. Rev. 40 (2011) 2279–2292.
[23] G.J. Kubas, Fundamentals of H2 binding and reactivity on transition metals underlying hydrogenase function and H2 production and storage, Chem. Rev. 107 (2007) 4152–4205.
[24] A.L. De Lacey, V.M. Fernandez, M. Rousset, R. Cammack, Activation and inactivation of hydrogenase function and the catalytic cycle: spectroelectrochemical studies, Chem. Rev. 107 (2007) 4304–4330.
[25] D.C. Brune, Sulfur oxidation by phototrophic bacteria, Biochim. Biophys. Acta 975 (1989) 189–221.
[26] A.A. DiSpirito, O.H. Tuovinen, Uranous ion oxidation and carbon dioxide fixation by *Thiobacillus ferrooxidans*, Arch. Microbiol. 133 (1982) 28–32.
[27] A. Elbehti, G. Brasseur, D. Lemesle-Meunier, First evidence for existence of an uphill electron transfer through the bc(1) and NADH-Q oxidoreductase complexes of the acidophilic obligate chemolithotrophic ferrous ion-oxidizing bacterium *Thiobacillus ferrooxidans*, J. Bacteriol. 182 (2000) 3602–3606.
[28] S. Klamt, H. Grammel, R. Straube, R. Ghosh, E.D. Gilles, Modeling the electron transport chain of purple non-sulfur bacteria, Mol. Syst. Biol. 4 (2008) 156.
[29] A.H. Stouthamer, A.P. de Boer, J. van der Oost, R.J. van Spanning, Emerging principles of inorganic nitrogen metabolism in *Paracoccus denitrificans* and related bacteria, Anton. Leeuw. 71 (1997) 33–41.
[30] D.T. Whipple, J.A. Kenis, Prospects of CO2 utilization via direct heterogeneous electrochemical reduction, J. Phys. Chem. Lett. 1 (2010) 3451–3458.
[31] W. Li, Electrocatalytic reduction of CO2 to small organic molecule fuels on metal catalysts, in: Advances in CO2 Conversion and Utilization, 2010, pp. 55–76.
[32] K.P. Kuhl, E.R. Cave, D.N. Abram, T.F. Jaramillo, New insights into the electrochemical reduction of carbon dioxide on metallic copper surfaces, Energy Environ. Sci. 5 (2012) 7050–7059.
[33] E.A. Quadrelli, G. Centi, J.L. Duplan, S. Perathoner, Carbon dioxide recycling: emerging large-scale technologies with industrial potential, Chemsuschem 4 (2011) 1194–1215.
[34] T. Reda, C.M. Plugge, N.J. Abram, J. Hirst, Reversible interconversion of carbon dioxide and formate by an electroactive enzyme, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 10654–10658.
[35] R.P.S. Chaplin, A.A. Wragg, Effects of process conditions and electrode material on reaction pathways for carbon dioxide electroreduction with particular reference to formate formation, J. Appl. Electrochem. 33 (2003) 1107–1123.
[36] B. Innocent, D. Liaigre, D. Pasquier, F. Ropital, J.-M. Léger, K.B. Kokoh, Electro-reduction of carbon dioxide to formate on lead electrode in aqueous medium, J. Appl. Electrochem. 39 (2009) 227–232.
[37] C. Oloman, H. Li, Electrochemical processing of carbon dioxide, Chemsuschem 1 (2008) 385–391.
[38] S. Enthaler, J. von-Langermann, T. Schmidt, Carbon dioxide and formic acid—the couple for environmental-friendly hydrogen storage ? Energy Environ. Sci. 3 (2010) 1207–1217.
[39] J.H. Hull, Y. Himeda, W.-H. Wang, B. Hashiguchi, R. Periana, D.J. Szalda, J.T. Muckerman, E. Fujita, Reversible hydrogen storage using CO2 and a proton-switchable iridium catalyst in aqueous media under mild temperatures and pressures, Nat. Chem. 4 (2012) 383–388.
[40] F. Joo, Breakthroughs in hydrogen storage—formic acid as a sustainable storage material for hydrogen, Chemsuschem 1 (2008) 805–808.
[41] B. Loges, A. Boddien, F. Gärtner, H. Junge, M. Beller, Catalytic generation of hydrogen from formic acid and its derivatives: useful hydrogen storage materials, Top. Catal. 53 (2010) 902–914.
[42] M.E. Lidstrom, Aerobic Methylotrophic Prokaryotes, in: M. Dworkin, S. Falkow, E. Rosenberg, K.H. Schleifer, E. Stackebrandt (Eds.), The Prokaryotes, Springer, New York, 2006, pp. 618–634.
[43] J. Schrader, M. Schilling, D. Holtmann, D. Sell, M.V. Filho, A. Marx, J.A. Vorholt, Methanol-based industrial biotechnology: current status and future perspectives of methylotrophic bacteria, Trends Biotechnol. 27 (2009) 107–115.
[44] L. Chistoserdova, M.G. Kalyuzhnaya, M.E. Lidstrom, The expanding world of methylotrophic metabolism, Annu. Rev. Microbiol. 63 (2009) 477–499.
[45] C. Anthony, The Biochemistry of Methylotrophs, Academic Press, London, 1982.
[46] H. Li, P.H. Opgenorth, D.G. Wernick, S. Rogers, T.Y. Wu, W. Higashide, P. Malati, Y.X. Huo, K.M. Cho, J.C. Liao, Integrated electromicrobial conversion of CO2 to higher alcohols, Science 335 (2012) 1596.
[47] A. Bar-Even, E. Noor, N.E. Lewis, R. Milo, Design and analysis of synthetic carbon fixation pathways, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 8889–8894.
[48] A. Flamholz, E. Noor, A. Bar-Even, R. Milo, eQuilibrator—the biochemical thermodynamics calculator, Nucleic Acids Res. 40 (2011) D700–D775.
[49] A. Bar-Even, E. Noor, R. Milo, A survey of carbon fixation pathways through a quantitative lens, J. Exp. Bot. 63 (2012) 2325–2342.
[50] E. Noor, A. Bar-Even, A. Flamholz, Y. Lubling, D. Davidi, R. Milo, An integrated open framework for thermodynamics of reactions that combines accuracy and coverage, Bioinformatics 28 (2012) 2037–2044.
[51] N.R. Boyle, J.A. Morgan, Computation of metabolic fluxes and efficiencies for biological carbon dioxide fixation, Metab. Eng. 13 (2011) 150–158.
[52] A. Walter, J. Gutknecht, Permeability of small nonelectrolytes through lipid bilayer membranes, J. Membr. Biol. 90 (1986) 207–217.
[53] T.X. Xiang, B.D. Anderson, The relationship between permeant size and permeability in lipid bilayer membranes, J. Membr. Biol. 140 (1994) 111–122.
[54] Y. Wang, Y. Huang, J. Wang, C. Cheng, W. Huang, P. Lu, Y.N. Xu, P. Wang, N. Yan, Y. Shi, Structure of the formate transporter FocA reveals a pentameric aquaporin-like channel, Nature 462 (2009) 467–472.
[55] I. Yamamoto, T. Saiki, S.M. Liu, L.G. Ljungdahl, Purification and properties of NADP-dependent formate dehydrogenase from *Clostridium thermoaceticum*, a tungsten-selenium-iron protein, J. Biol. Chem. 258 (1983) 1826–1832.
[56] A.P. Shuber, E.C. Orr, M.A. Recny, P.F. Schendel, H.D. May, N.L. Schauer, J.G. Ferry, Cloning, expression, and nucleotide sequence of the formate dehydrogenase genes from *Methanobacterium formicicum*, J. Biol. Chem. 261 (1986) 12942–12947.
[57] J. Friedebold, B. Bowien, Physiological and biochemical characterization of the soluble formate dehydrogenase, a molybdoenzyme from *Alcaligenes eutrophus*, J. Bacteriol. 175 (1993) 4719–4728.
[58] H. Raaijmakers, S. Teixeira, J.M. Dias, M.J. Almendra, C.D. Brondino, I. Moura, J.J. Moura, M.J. Romao, Tungsten-containing formate dehydrogenase from *Desulfovibrio gigas*: metal identification and preliminary structural data by multi-wavelength crystallography, J. Biol. Inorg. Chem. 6 (2001) 398–404.
[59] R.P. Schmitz, G. Diekert, Purification and properties of the formate dehydrogenase and characterization of the fdhA gene of *Sulfurospirillum multivorans*, Arch. Microbiol. 180 (2003) 394–401.
[60] M. Laukel, L. Chistoserdova, M.E. Lidstrom, J.A. Vorholt, The tungsten-containing formate dehydrogenase from *Methylobacterium extorquens* AM1: purification and properties, Eur. J. Biochem. 270 (2003) 325–333.
[61] V.I. Tishkov, V.O. Popov, Catalytic mechanism and application of formate dehydrogenase, Biochemistry (Mosc) 69 (2004) 1252–1267.
[62] V.I. Tishkov, V.O. Popov, Protein engineering of formate dehydrogenase, Biomol. Eng. 23 (2006) 89–110.
[63] H.G. Enoch, R.L. Lester, The purification and properties of formate dehydrogenase and nitrate reductase from Escherichia coli, J. Biol. Chem. 250 (1975) 6693–6705.
[64] B.L. Berg, J. Li, J. Heider, V. Stewart, Nitrate-inducible formate dehydrogenase in *Escherichia coli* K-12. I. Nucleotide sequence of the fdnGHI operon and evidence that opal (UGA) encodes selenocysteine, J. Biol. Chem. 266 (1991) 22380–22385.
[65] I.A. Berg, Ecological aspects of the distribution of different autotrophic CO2 fixation pathways, Appl. Environ. Microbiol. 77 (2011) 1925–1936.
[66] G. Fuchs, Alternative pathways of carbon dioxide fixation — insights into the early evolution of life, Annu. Rev. Microbiol. 65 (2011) 631–658.
[67] A. Bar-Even, A. Flamholz, E. Noor, R. Milo, Thermodynamic constraints shape the structure of carbon fixation pathways, Biochim. Biophys. Acta 1817 (9) (2012) 1646–1659.
[68] C.G. Friedrich, B. Friedrich, B. Bowien, Formation of enzymes of autotrophic metabolism during heterotrophic growth of *Alcaligenes eutrophus*, Microbiology 122 (1980) 69–78.
[69] H.W. van Verseveld, A.H. Stouthamer, Growth yields and the efficiency of oxidative phosphorylation during autotrophic growth of *Paracoccus denitrificans* on methanol and formate, Arch. Microbiol. 118 (1978) 21–26.
[70] D.P. Kelly, A.P. Wood, J.C. Gottschal, J.G. Kuenen, Autotrophic metabolism of formate by thiobacillus strain a2, Microbiology 114 (1979) 1–13.
[71] L. Dijkhuizen, M. Knight, W. Harder, Metabolic regulation in *Pseudomonas oxalaticus* OX1. Autotrophic and heterotrophic growth on mixed substrates, Arch. Microbiol. 116 (1978) 77–83.

[72] I. Goldberg, J.S. Rock, A. Ben-Bassat, R.I. Mateles, Bacterial yields on methanol, methylamine, formaldehyde, and formate, Biotechnol. Bioeng. 18 (1976) 1657–1668.
[73] S. Vuilleumier, L. Chistoserdova, M.C. Lee, F. Bringel, A. Lajus, Y. Zhou, B. Gourion, V. Barbe, J. Chang, S. Cruveiller, C. Dossat, W. Gillett, C. Gruffaz, E. Haugen, E. Hourcade, R. Levy, S. Mangenot, E. Muller, T. Nadalig, M. Pagni, C. Penny, R. Peyraud, D.G. Robinson, D. Roche, Z. Rouy, C. Saenampechek, G. Salvignol, D. Vallenet, Z. Wu, C.J. Marx, J.A. Vorholt, M.V. Olson, R. Kaul, J. Weissenbach, C. Medigue, M.E. Lidstrom, Methylobacterium genome sequences: a reference blueprint to investigate microbial metabolism of C1 compounds from natural and industrial sources, PLoS One 4 (2009) e5584.
[74] K. Jansen, R.K. Thauer, F. Widdel, G. Fuchs, Carbon assimilation pathways in sulfate reducing bacteria. Formate, carbon dioxide, carbon monoxide, and acetate assimilation by *Desulfovibrio baarsii*, Arch. Microbiol. 138 (1984) 257–262.
[75] M.M. Attwood, W. Harder, Formate assimilation by Hyphomicrobium X, FEMS Microbiol. Lett. 3 (1978) 111–114.
[76] R. Peyraud, P. Kiefer, P. Christen, S. Massou, J.C. Portais, J.A. Vorholt, Demonstration of the ethylmalonyl-CoA pathway by using 13C metabolomics, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 4846–4851.
[77] C. Anthony, How half a century of research was required to understand bacterial growth on C1 and C2 compounds; the story of the serine cycle and the ethylmalonyl-CoA pathway, Sci. Prog. 94 (2011) 109–137.
[78] T.J. Erb, I.A. Berg, V. Brecht, M. Muller, G. Fuchs, B.E. Alber, Synthesis of C5-dicarboxylic acids from C2-units involving crotonyl-CoA carboxylase/reductase: the ethylmalonyl-CoA pathway, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 10631–10636.
[79] L. Chistoserdova, S.W. Chen, A. Lapidus, M.E. Lidstrom, Methylotrophy in *Methylobacterium extorquens* AM1 from a genomic point of view, J. Bacteriol. 185 (2003) 2980–2987.
[80] L. Chistoserdova, Modularity of methylotrophy, revisited, Environ. Microbiol. 13 (2011) 2603–2622.
[81] H.L. Drake, K. Kirsten, C. Matthies, Acetogenic prokaryotes, in: The Prokaryotes, Springer, New York, 2006, pp. 354–420.
[82] H.L. Drake, A.S. Gossner, S.L. Daniel, Old acetogens, new light, Ann. N. Y. Acad. Sci. 1125 (2008) 100–128.
[83] S.W. Ragsdale, Enzymology of the wood-Ljungdahl pathway of acetogenesis, Ann. N. Y. Acad. Sci. 1125 (2008) 129–136.
[84] S.W. Ragsdale, E. Pierce, Acetogenesis and the Wood–Ljungdahl pathway of CO(2) fixation, Biochim. Biophys. Acta 1784 (2008) 1873–1898.
[85] M.A. Gleeson, P.E. Sudbery, The methylotrophic yeasts, Yeast 4 (1988) 1–15.
[86] L.J. Waber, H.G. Wood, Mechanism of acetate synthesis from CO2 by *Clostridium acidiurici*, J. Bacteriol. 140 (1979) 468–478.
[87] P. Durre, J.R. Andreesen, Pathway of carbon dioxide reduction to acetate without a net energy requirement in *Clostridium purinolyticum*, FEMS Microbiol. Lett. 15 (1982) 51–56.
[88] H. Schiefer-Ullrich, R. Wagner, P. Durre, J.R. Andreesen, Comparative studies on physiology and taxonomy of obligately purinolytic clostridia, Arch. Microbiol. 138 (1984) 345–353.
[89] N. Fonknechten, S. Chaussonnerie, S. Tricot, A. Lajus, J.R. Andreesen, N. Perchat, E. Pelletier, M. Gouyvenoux, V. Barbe, M. Salanoubat, D. Le Paslier, J. Weissenbach, G.N. Cohen, A. Kreimeyer, Clostridium sticklandii, a specialist in amino acid degradation:revisiting its metabolism through its genome sequence, BMC Genomics 11 (2010) 555.
[90] P. Durre, R. Spahr, J.R. Andreesen, Glycine fermentation via a glycine reductase in *Peptococcus glycinophilus* and *Peptococcus magnus*, Arch. Microbiol. 134 (1983) 127–135.
[91] A. Schneeberger, J. Frings, B. Schink, Net synthesis of acetate from CO2 by *Eubacterium acidaminophilum* through the glycine reductase pathway, FEMS Microbiol. Lett. 177 (1999).
[92] G. Fuchs, CO2 fixation in acetogenic bacteria: variations on a theme, FEMS Microbiol. Lett. 39 (1985) 181–213.
[93] J.R. Andreesen, Glycine reductase mechanism, Curr. Opin. Chem. Biol. 8 (2004) 454–461.
[94] R. Braakman, E. Smith, The emergence and early evolution of biological carbon-fixation, PLoS Comput. Biol. 8 (2012) e1002455.
[95] L.B. Pasternack, D.A. Laude Jr., D.R. Appling, 13C NMR detection of folate-mediated serine and glycine synthesis in vivo in *Saccharomyces cerevisiae*, Biochemistry 31 (1992) 8713–8719.
[96] Q. Zhang, J.T. Wiskich, Activation of glycine decarboxylase in pea leaf mitochondria by ATP, Arch. Biochem. Biophys. 320 (1995) 250–256.
[97] H. Maaheimo, J. Fiaux, Z.P. Cakar, J.E. Bailey, U. Sauer, T. Szyperski, Central carbon metabolism of *Saccharomyces cerevisiae* explored by biosynthetic fractional (13) C labeling of common amino acids, Eur. J. Biochem. 268 (2001) 2464–2479.
[98] H. Kawasaki, T. Sato, G. Kikuchi, A new reaction for glycine biosynthesis, Biochem. Biophys. Res. Commun. 23 (1966) 227–233.
[99] G. Kikuchi, The glycine cleavage system: composition, reaction mechanism, and physiological significance, Mol. Cell. Biochem. 1 (1973) 169–187.
[100] H. Kochi, G. Kikuchi, Mechanism of reversible glycine cleavage reaction in *Arthrobacter globiformis*. Function of lipoic acid in the cleavage and synthesis of blycine, Arch. Biochem. Biophys. 173 (1976) 71–81.
[101] C. Anthony, The *Prediction of Growth Yields in Methylotrophs*, Microbiology 104 (1978) 91–104.
[102] K.B. Andersen, K. von Meyenburg, Are growth rates of Escherichia coli in batch cultures limited by respiration? J. Bacteriol. 144 (1980) 114–123.
[103] Y. Noguchi, Y. Nakai, N. Shimba, H. Toyosaki, Y. Kawahara, S. Sugimoto, E. Suzuki, The energetic conversion competence of *Escherichia coli* during aerobic respiration studied by 31P NMR using a circulating fermentation system, J. Biochem. 136 (2004) 509–515.
[104] J.D. Orth, I. Thiele, B.O. Palsson, What is flux balance analysis? Nat. Biotechnol. 28 (2010) 245–248.
[105] K. Raman, N. Chandra, Flux balance analysis of biological systems: applications and challenges, Brief. Bioinform. 10 (2009) 435–449.
[106] J.D. Orth, R.M.T. Fleming, B.O. Palsson, The Core *E. coli* Model, http://gcrg.ucsd.edu/Downloads/EcoliCore2009.
[107] A.M. Feist, B.O. Palsson, The biomass objective function, Curr. Opin. Microbiol. 13 (2010) 344–349.
[108] R.A. Alberty, Thermodynamics of Biochemical Reactions, Wiley-Interscience, 2003.
[109] M.L. Mavrovouniotis, Identification of localized and distributed bottlenecks in metabolic pathways, Proc. Int. Conf. Intell. Syst. Mol. Biol. 1 (1993) 275–283.
[110] E.L. Maynard, P.A. Lindahl, Evidence of a molecular tunnel connecting the active sites for CO2 reduction and acetyl-CoA synthesis in acetyl-CoA synthase from *Clostridium thermoaceticum*, J. Am. Chem. Soc. 121 (1999) 9221–9222.
[111] J. Seravalli, S.W. Ragsdale, Channeling of carbon monoxide during anaerobic carbon dioxide fixation, Biochemistry 39 (2000) 1274–1277.
[112] T.I. Doukov, L.C. Blasiak, J. Seravalli, S.W. Ragsdale, C.L. Drennan, Xenon in and at the end of the tunnel of bifunctional carbon monoxide dehydrogenase/acetyl-CoA synthase, Biochemistry 47 (2008) 3474–3483.
[113] B.D. Bennett, E.H. Kimball, M. Gao, R. Osterhout, S.J. Van Dien, J.D. Rabinowitz, Absolute metabolite concentrations and implied enzyme active site occupancy in Escherichia coli, Nat. Chem. Biol. 5 (2009) 593–599.
[114] A. Bar-Even, E. Noor, A. Flamholz, J.M. Buescher, R. Milo, Hydrophobicity and charge shape cellular metabolite concentrations, PLoS Comput. Biol. 7 (2011) e1002166.
[115] H. Qian, D.A. Beard, S.D. Liang, Stoichiometric network theory for nonequilibrium biochemical systems, Eur. J. Biochem. 270 (2003) 415–421.
[116] D.A. Beard, H. Qian, Relationship between thermodynamic driving force and one-way fluxes in reversible processes, PLoS One 2 (2007) e144.
[117] A. Bar-Even, A. Flamholz, E. Noor, R. Milo, Rethinking glycolysis: on the biochemical logic of metabolic pathways, Nat. Chem. Biol. 8 (2012) 509–517.
[118] H. Bremer, P. Dennis, Modulation of chemical composition and other parameters of the cell by growth rate, in: Escherichia coli and Salmonella, 1987.
[119] W.G. Meijer, L. Dijkhuizen, Regulation of autotrophic metabolism in *Pseudomonas oxalaticus* OX1 wild-type and an isocitrate-lyase-deficient mutant, J. Gen. Microbiol. 134 (1988) 3231–3237.
[120] R.G. Kallen, W.P. Jencks, The mechanism of the condensation of formaldehyde with tetrahydrofolic acid, J. Biol. Chem. 241 (1966) 5851–5863.
[121] B. Volkmer, M. Heinemann, Condition-dependent cell volume and concentration of *Escherichia coli* to facilitate data conversion for systems biology modeling, PLoS One 6 (2011) e23126.
[122] M. Loferer-Krossbacher, J. Klima, R. Psenner, Determination of bacterial cell dry mass by transmission electron microscopy and densitometric image analysis, Appl. Environ. Microbiol. 64 (1998) 688–694.
[123] G.J. Crowther, G. Kosaly, M.E. Lidstrom, Formate as the main branch point for methylotrophic metabolism in *Methylobacterium extorquens* AM1, J. Bacteriol. 190 (2008) 5057–5062.
[124] R.J. Wilkins, H.D. Macleod, Formaldehyde induced DNA-protein crosslinks in *Escherichia coli*, Mutat. Res. 36 (1976) 11–16.
[125] B.K. Hunter, K.M. Nicholls, J.K. Sanders, Formaldehyde metabolism by *Escherichia coli*. In vivo carbon, deuterium, and two-dimensional NMR observations of multiple detoxifying pathways, Biochemistry 23 (1984) 508–514.
[126] S.M. Klein, R.D. Sagers, Glycine metabolism. II. Kinetic and optical studies on the glycine decarboxylase system from *Peptococcus glycinophilus*, J. Biol. Chem. 241 (1966) 206–209.
[127] K. Hiraga, G. Kikuchi, The mitochondrial glycine cleavage system. Functional association of glycine decarboxylase and aminomethyl carrier protein, J. Biol. Chem. 255 (1980) 11671–11676.
[128] K. Fujiwara, Y. Motokawa, Mechanism of the glycine cleavage reaction. Steady state kinetic studies of the P-protein-catalyzed reaction, J. Biol. Chem. 258 (1983) 8156–8162.
[129] K. Okamura-Ikeda, K. Fujiwara, Y. Motokawa, The amino-terminal region of the *Escherichia coli* T-protein of the glycine cleavage system is essential for proper association with H-protein, Eur. J. Biochem. 264 (1999) 446–453.
[130] K. Okamura-Ikeda, N. Kameoka, K. Fujiwara, Y. Motokawa, Probing the H-protein-induced conformational change and the function of the N-terminal region of *Escherichia coli* T-protein of the glycine cleavage system by limited proteolysis, J. Biol. Chem. 278 (2003) 10067–10072.
[131] M. Aoshima, Y. Igarashi, A novel oxalosuccinate-forming enzyme involved in the reductive carboxylation of 2-oxoglutarate in *Hydrogenobacter thermophilus* TK-6, Mol. Microbiol. 62 (2006) 748–759.
[132] M. Aoshima, M. Ishii, Y. Igarashi, A novel biotin protein required for reductive carboxylation of 2-oxoglutarate by isocitrate dehydrogenase in *Hydrogenobacter thermophilus* TK-6, Mol. Microbiol. 51 (2004) 791–798.
[133] J.A. Vorholt, C.J. Marx, M.E. Lidstrom, R.K. Thauer, Novel formaldehyde-activating enzyme in *Methylobacterium extorquens* AM1 required for growth on methanol, J. Bacteriol. 182 (2000) 6645–6650.
[134] N.K. Ramaswamy, A.G. Behere, P.M. Nair, A novel pathway for the synthesis of solanidine in the isolated chloroplast from greening potatoes, Eur. J. Biochem. 67 (1976) 275–282.
[135] M.T. Janave, N.K. Ramaswamy, P.M. Nair, Purification and characterization of glyoxylate synthetase from greening potato-tuber chloroplasts, Eur. J. Biochem. 214 (1993) 889–896.
[136] M.T. Janave, N.K. Ramaswamy, P.M. Nair, Studies on determination of active site amino acid residues in glyoxylate synthetase from potato tuber chloroplasts, Plant Physiol. Biochem. 37 (1999) 121–129.